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PROGRESS IN CHROMATOGRAPHY,
ELECTROPHORESIS AND RELATED METHODS

Volume 1

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PREFACE

In recent years not only have chromatographic and electrophoretic methods been used to separate most classes of organic and inorganic compounds, but also numerous new fields of research have become possible through their use.

We believe that today a monograph on chromatographic and electrophoretic methods can be written only by a team of workers who specialise in various fields.

In founding the *Journal of Chromatography* plans were made to present critical reviews on separation methods, written by specialists. However, a journal appears only in a limited edition, and the numerous requests for reprints received by the authors have indicated that these reviews are of interest to a wider circle of readers. It was thus decided to publish the reviews in book form. While the *Journal of Chromatography* contains reviews in English, French and German, this book is entirely in English, the reviews in French and German having been translated into English. (Part of this volume, the review by NEHER, has already appeared in book form in German; R. NEHER, *Chromatographie von Sterinen, Steroiden und verwandten Verbindungen*, Elsevier, Amsterdam, 1958.)

The various chapters were either invited or selected so that the topics complete and enlarge upon the monographs *Chromatography, A Review of Principles and Applications*, by E. LEDERER and M. LEDERER (Elsevier, Amsterdam, 2nd Edition, 1957) and *Introduction to Paper Electrophoresis and Related Methods*, by M. LEDERER (Elsevier, Amsterdam, 2nd Impression, 1957). Altogether there are two chapters on general techniques, two on inorganic separations and the rest on organic compounds. The chapter by Dr. REIO on the paper chromatography of phenol derivatives is actually original work, but it was included in this volume because of its length and the amount of new data presented.

The next volume will deal with gas-liquid chromatography, gas-adsorption chromatography, starch gel electrophoresis, techniques of measuring radioactivity on paper chromatograms, the separation of DNP-amino acids, inorganic adsorption chromatography and other topics.

The editor would be grateful for suggestions as to other fields in which further reviews would be helpful.

Paris, January 1959 MICHAEL LEDERER

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CHROMATOSTRIPS AND CHROMATOPLATES

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1. CHROMATOSTRIPS

In 1951, KIRCHNER, MILLER AND KELLER[1] described a technique of adsorption microchromatography in which strips of glass are used to support a thin layer of mineral adsorbent ("chromatostrips"), this being a modification of the radial chromatography of inorganic ions suggested by MEINHARD AND HALL[2].

A chromatostrip is usually prepared by coating one side of a glass strip with a paste consisting of an adsorbent, water and a binding agent such as starch. After drying the adsorbent layer hardens to a thickness of about 0.5 mm. One ascending microchromatogram can be carried out on each strip, a few μg of the substance to be examined being spotted near one end of the strip, which is then dipped into the solvent chosen for the development. The strip is held vertically, for example in a test-tube of convenient size. The rapid capillary rise permits a development of only about 30 minutes. After development it only remains to note the positions of the constituents of the chromatographed substance, found as spots between the starting point of the microchromatogram and the limit of the solvent front. If, as frequently happens, colourless substances are chromatographed, colour reactions are used to detect the spots ("location").

It should be emphasized here that the separations obtained in chromatostrips are the result of a "true" adsorption process, and hence differ essentially from the technique of paper chromatography, which is based on a partition mechanism.

2. CHROMATOPLATES

All reliable chromatographic methods must yield precise and reproducible results. However, separations on chromatostrips are strongly influenced by the degree of activation of the adsorbent as well as by its thickness; these two factors must be kept constant and this is achieved by the use of a special apparatus which can produce layers of known thickness[3,4] and by drying the layers under standard conditions. In this way, separations reproducible to $\pm$ 0.05 R_F can be obtained[5] provided that the chromatostrips are protected from atmospheric humidity during the procedure, this proving sometimes rather difficult.

The method was considerably simplified in 1954 by REITSEMA[6], who used *chromatoplates* or large chromatostrips on which several microchromatograms can be done

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simultaneously. The different separations obtained on the same plate are strictly comparable so long as the adsorbent is distributed very regularly.

Comparison of chromatography on paper and on chromatoplates

These two procedures, besides their common qualities, present different possibilities which accord with the physico-chemical mechanisms which characterize them (partition or "true" adsorption). For example, some main applications of paper chromatography (separations of sugars and amino acids) do not seem practicable on chromatoplates. The same is true for the fractionation of homologous series, which is relatively simple on paper since the partition coefficients vary regularly with the increase in length of the carbon chains.

Chromatoplates are best applied to lipid-soluble materials which are usually of moderate polarity. They have several advantages among which the following may be mentioned. The procedure is simple and rapid; the solvent, chosen for its eluting properties as in the case of column chromatography, is homogeneous; the surrounding temperature and the amount of substance chromatographed are without effect within reasonable limits; the development of the chromatoplates takes place in a remarkably short time (usually 15–30 min); and finally, the adsorbent, which can be varied at need, withstands the action of strong detecting reagents.

The possibility of adapting chromatoplates to the principle of partition chromatography should not be excluded. At present, this method is suitable for control work or as a rapid and flexible means of preliminary investigation. Although paper chromatography is sometimes less easy to apply, it produces R_F-values of higher precision owing to the longer development possible.

3. APPLICATIONS OF CHROMATOPLATES

The original papers of KIRCHNER *et al.*[1,5,7–10] give a good idea of the scope of the method as applied to the examination and identification of various compounds, often of the terpene type, in essential oils. More recently, other workers have used it for the same purpose[3,11–15] and ONOE[16] has employed it for the separation of dinitrophenylhydrazones of aliphatic aldehydes. REITSEMA[6] also made a comparative study of a series of essential oils using the chromatoplates which he devised, and WAGNER[17] examined several phenols by the same technique. Other publications mention the use of chromatostrips for the separation of azulenes[3,27] and for the study of organic peroxides and coumarins[29]. These applications together with the examples of our own (unpublished) results given below illustrate the general applicability of the method.

Adsorbents and solvents generally used

In the field of essential oils, silicic acid is the most widely used adsorbent on chromatoplates. The silicic layers not only possess excellent mechanical properties (hardness, etc.) but also show a remarkable selectivity towards the oxygenated components of

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the essential oils. This selectivity can be explained by an adsorption mechanism involving the formation of hydrogen bonds between silicic acid and the adsorbed compound[18,19]; molecules containing atoms with free electronic doublets (O, N) are particularly sensitive to this mechanism and are more or less retained according to the polarity and the steric hindrance of the active centres.

In general, silicic acid is very suitable for handling sensitive materials. Using this adsorbent in columns as well as on chromatoplates, the writer has chromatographed acyclic, diterpenic allyl alcohols[20]; the separations were good and there was no sign of dehydration or isomerization. However, in certain cases it may be preferable to de-activate the silicic acid partially before use[21].

KIRCHNER *et al.*[1] described several mixtures of solvents for the development stage. As in all adsorption chromatography work, the eluting properties of the solvent and the affinity of the substance for the adsorbent, which are established by preliminary tests, must be considered. In the field of essential oils, mixtures of ethyl acetate with hexane or benzene are often applied with excellent results; 10–20% of ethyl acetate is generally present.

Methods of detection

KIRCHNER *et al.* (*loc. cit.*) suggested various methods of detection according to the nature of the adsorbed material. Thus, they detected unsaturated compounds by their "masking effect" on a fluorescent adsorbent, the principle being similar to that of the technique of SEASE[22]; the spots appear as shadows against a brilliant background under ultraviolet light of short wavelength. REITSEMA[6] also utilized the possibilities of ultraviolet light. Obviously, the detection of coloured or fluorescent compounds does not present any problem.

A chemical method of detection of unsaturated compounds, involving the combined action of fluorescein and bromine vapour, has also been described by KIRCHNER *et al.*[1]. In addition, these authors used *o*-dianisidine in acetic acid solution to detect aldehydes, and bromocresol green, previously recommended by RAMSEY AND PATTERSON[23], for acids. They located particularly inert organic compounds by treatment with cold concentrated sulphuric acid or even with a hot mixture of sulphuric and nitric acids; in these cases, the starch normally used as binding agent in the adsorbent layers was replaced by plaster.

Other chemical reactions can be devised for detection purposes according to the functional type of the chromatographed substance. Thus, ketones and aldehydes have been detected by reaction with 2,4-dinitrophenylhydrazine in hydrochloric acid solution[6], and phenols by coupling with diazotized sulphanilic acid[17]. Some unpublished reactions have been applied by the writer. Very dilute solutions of potassium permanganate, or iodine vapour at high temperatures, are suitable for locating compounds which are *unsaturated* or readily dehydrated. *Phenols* can be detected by diazotized *p*-nitroaniline prepared according to RAYBURN *et al.*[24], or by ferric chloride in water–alcohol solution. Diazotized *p*-nitraniline is also satisfactory for the detection of any compound, such as *indole* (red colour), which can couple with it. As in paper chromatog-

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raphy, ninhydrin can be applied to locate *primary amines*[25]. In the experimental part below is described the detection of *volatile esters* by an adaptation of the hydroxamic acid reaction described by FEIGL[26].

All these reactions allow the use of chromatoplates as a qualitative or semi-quantitative method of analysis. KIRCHNER *et al.*[9] have shown that it is also possible to adapt their technique to obtain quantitative results.

Microchemical reactions on chromatostrips or chromatoplates

The reproducibility of R_F-values on chromatostrips is approximately ± 0.05[5]. However, this excellent precision does not permit the formal identification of the components of the essential oils on the basis of the R_F-values alone, even when they are determined in several solvents. This is the reason why MILLER AND KIRCHNER[5] degraded or prepared derivatives of some substances before microchromatography; the new products permitted a more certain characterization of the starting material. Some of these reactions can be carried out directly on the adsorbent by covering the substance with a drop of reagent before starting the development. Oxidations (CrO_3), dehydrations (H_2SO_4), reductions ($LiAlH_4$) and preparations of semicarbazone and phenylhydrazone derivatives have been done on chromatostrips in this way.

Some other reactions, *e.g.* hot alkaline hydrolysis and urethan formation, are preferably carried out in micro-tubes; in this case a drop of the raw reaction product is chromatographed.

A series of natural products can be characterized by means of these microchemical reactions. For example, citral can be oxidized to geranic acid by hydrogen peroxide and the acid reduced to geraniol, this sequence ensuring correct identification.

Application of chromatoplates to chromatography on the preparative scale

Compounds containing all the principal functional groups have been chromatographed on chromatoplates and any material of low polarity and suitable solubility can in principle be examined even when it is relatively volatile as in the case of butyric aldehyde[6].

Moreover, microchromatograms which are successful on chromatoplates can be reproduced on a column of the same adsorbent on the preparative scale. Thus a rapid method is available for the exploration of suitable conditions for large scale chromatograms which only sacrifices small quantities of material[1,7]. However, it should be remembered that, other conditions being equal, columns are rather less selective than chromatoplates, due to diffusion of bands on the former. Besides, some solvent mixtures undergo "demixion" (caused by "frontal chromatography") on chromatoplates and this phenomenon, which can have a favorable influence on certain separations, is not reproduced on columns prepared by sedimentation of the adsorbent in an excess of solvent.

The progress of a liquid chromatogram can be followed exactly by controlling each eluate on a chromatoplate; it is thus possible to distinguish rapidly between

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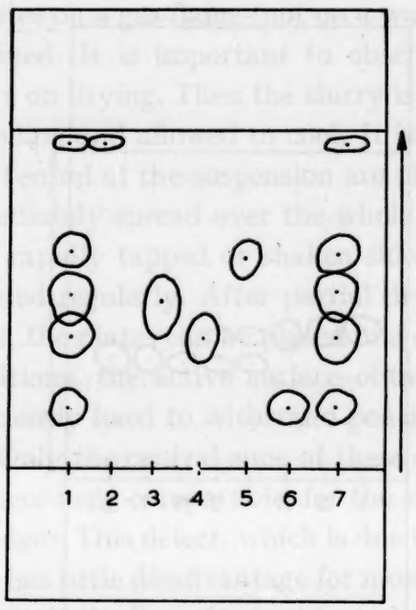

Fig. 1.

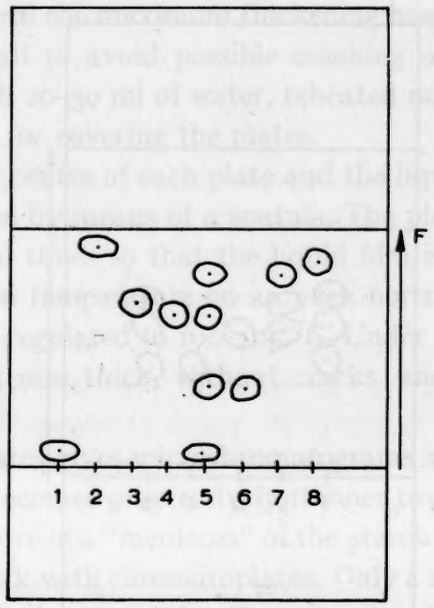

Fig. 2.

Fig. 1. Separation of carotenoids on chromatoplates. 5–10 μg spotted in acetone solution. 2 = β-Carotene R_F 0.96; 3 = Bixin R_F 0.51; 4 = Canthaxanthin R_F 0.38; 5 = Isozeaxanthin R_F 0.63; 6 = Zeaxanthin R_F 0.17; 1 and 7 = Mixture. Adsorbent: silicic acid, 0.3 mm layer. Solvent: *n*-hexane–ether 3:7 (v/v). Distance covered by solvent front: 76 mm. Time of development: 15 minutes.

Fig. 2. Separation of tetrapyrrolic pigments on chromatoplates. Porphyrins (methyl esters) and bilirubin spotted in benzene solution; biliverdin in alcoholic solution. 1 = Biliverdin 3 μg R_F 0.06; 2 = Bilirubin 7 μg R_F 0.92; 3 = Coproporphyrin I ester, 7 μg R_F 0.68; 4 = Coproporphyrin III ester, 15 μg R_F 0.63; 6 = Uroporphyrin I ester, 9 μg R_F 0.33; 7 = Deuteroporphyrin ester 3 μg R_F 0.80; 8 = Protoporphyrin ester, 5 μg R_F 0.85; 5 = Mixture of 1, 4, 6, 7. Adsorbent: silicic acid, 0.3 mm layer. Solvent: benzene–ethyl acetate–ethyl alcohol, 90:20:7.5 (v/v). Distance covered by solvent front: 54 mm. Time of development: 15 minutes.

homogeneous fractions and intermediate mixtures[7]. An example of such a procedure is given below.

Use of chromatoplates on a micropreparative scale

It is possible to chromatograph up to 1 mg of material on chromatostrips with a specially thick adsorbent layer ($\sim$ 1 mm thick). After development, the adsorbent holding the various spots is cut out and extracted with an appropriate solvent; the spots are located by comparison with a second chromatostrip which is revealed chemically. The micro-eluates obtained from the spots contain sufficient material for spectrographic study or for further micro-work.

Naturally, thick chromatoplates are just as satisfactory as strips for these operations.

Conclusions

Figs. 1–4 illustrate the results obtained by the writer when chromatoplates were used for several separations in the field of carotenoids, tetrapyrrolic pigments and diterpenes.

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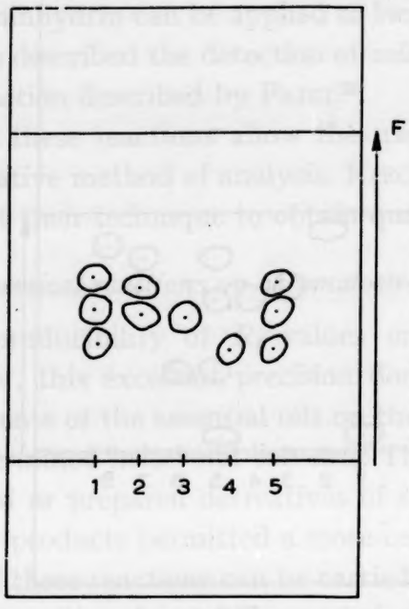

Fig. 3.

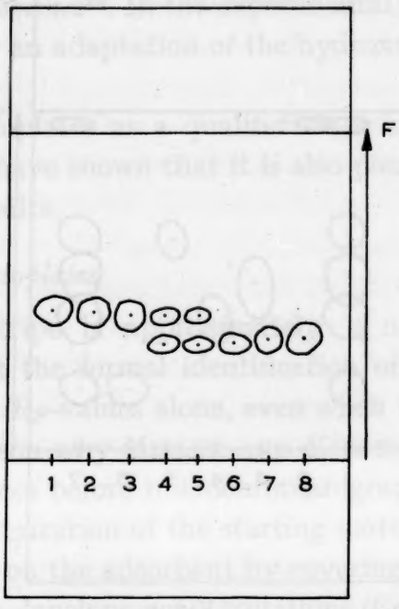

Fig. 4.

Fig. 3. Separation of diterpenoids of the phytol type on chromatoplates. 10 μg spotted in *n*-hexane solution. 1 and 5 = Mixture of isophytol, geranyl–linalol and phytol; 2 = Mixture of isophytol and geranyl–linalol; 3 = Geranyl–linalol; 4 = Phytol. Phytol R_F 0.34–0.35. Geranyl–linalol R_F 0.44–0.46. Isophytol R_F 0.53–0.55. Adsorbent: silicic acid, 0.3 mm layer. Solvent: ethyl acetate–*n*-hexane 1 : 4 (v/v). Distance covered by solvent front: 74 mm. Time of development: 15 minutes. Detection: 0.25% aqueous potassium permanganate solution.

Fig. 4. Control of a liquid chromatogram on a chromatoplate. A few μg of each of the 8 fractions resulting from a preparative column chromatogram were chromatographed on this plate. The process of fractionation is thus demonstrated clearly; fractions 4 and 5 are still mixtures. Mixture studied: phytol + geranyl–linalol. This chromatoplate was prepared under conditions similar to those of the preceding example.

The following experimental part contains detailed descriptions of the preparation and use of chromatoplates as well as some hitherto unpublished methods of detection of unsaturated compounds, phenols and esters.

It is the opinion of the author that the chromatoplate technique should be further examined and extended, for an analytical method of such simplicity which gives such valuable results is rarely encountered.

4. EXPERIMENTAL

Preparation of chromatoplates

Glass plates, 17 × 11 × 0.2 cm, and 100-mesh silicic acid (analytical reagent grade, Mallinckrodt Co.) were employed. The plates are coated according to the procedure of REITSEMA[6], save that the heating of the silicic mixture is slightly altered:

Water (54 ml) is added to a mixture of 28.5 g of silicic acid with 1.5 g of rice starch*. This is mixed together thoroughly and then heated near boiling for 2–3

* The type of starch is important.

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minutes on a gas flame (not on a water-bath) until the maximum thickening has been obtained. It is important to observe this detail to avoid possible cracking of the layers on drying. Then the slurry is diluted with 20–30 ml of water, reheated rapidly to boiling and allowed to cool. It is then ready for covering the plates.

Ten ml of the suspension are placed on the centre of each plate and the liquid is immediately spread over the whole glass surface by means of a spatula. The plate is then rapidly tapped or shaken sideways several times so that the liquid film is distributed regularly. After partial drying at room temperature on an even horizontal plane, the plates can be placed in a drying oven regulated to 100–105° C. Under these conditions, the active surface obtained are 0.5 mm thick, without cracks, and are sufficiently hard to withstand pencil writing.

Only the central zone of these chromatoplates gives microchromatograms which are rigorously comparable, for the silicic layer becomes progressively thinner towards the edges. This defect, which is due to the exposure of a "meniscus" of the starch mixture, has little disadvantage for most general work with chromatoplates. Only a slight increase in the R_F-value is observed on the marginal zones of the plates (border effect).

More uniform distribution of the adsorbent layer may be obtained if a special apparatus is used. For this purpose, the author prefers a commercial instrument normally destined for the production of thin layers of paint. This instrument which is placed horizontally consists of a sliding metal knife blade fixed at an adjustable distance above a plane surface. An excess of the starch mixture is placed on a glass plate fixed in this apparatus, the blade of which has previously been adjusted to a convenient height. When the blade is moved along, the excess of liquid is removed and only a film of the desired thickness remains. In this way, the writer obtains chromatoplates with a thickness of 0.3 mm on which the border effect is absent except at the extreme edges. The effect is entirely removed by scraping 10–15 mm of the adsorbent off each side of the plate.

Marking the chromatoplates

The chromatoplates are marked with a soft pencil on the well dried adsorbent. The starting points of the microchromatograms are spaced at 8–10 mm on a line situated 30 mm from the base of the plate. Up to 10 microchromatograms can be carried out on a plate of the above dimensions. After being marked, the plates are replaced in the oven for 30 minutes at 100–105° C for final activation. While still hot, they are placed in a desiccator containing potassium hydroxide or calcium chloride and are ready for use after cooling. The plates can be kept indefinitely if protected from moisture.

Application of the material to be chromatographed

The desiccator containing the chromatoplates should have a small opening at the top to allow the insertion of micropipettes holding solutions of the substances under study. It is thus possible to work while the plates are protected from atmospheric humidity. On each mark of the chromatoplate, 5–10 mm^3 of solution corresponding

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to an appropriate amount of material (generally 5–10 μg) are placed; the substance should be dissolved in a volatile solvent of slight polarity such as pentane, ether, etc.

Development of the chromatoplates

The chromatoplates are arranged in a small trough (21 × 12 × 4 cm) which is closed by a glass plate and contains 100 ml of solvent. The plates are held almost vertically and dip 2 cm into the liquid.

When the development is complete in 15–30 minutes, the solvent front has risen from 8 to 10 cm. Usually no advantage is gained by prolonging the development, for the spots begin to diffuse. Moreover, the flow of the solvent is initially very rapid but is quite slow after about 8 cm have been covered.

The plates are withdrawn and allowed to dry at a moderate temperature (50° C) after the position of the solvent front has been noted.

Solvents for development

Mixtures of ethyl acetate and hexane are employed frequently and are very suitable for general use. A proportion of 15–20% of ethyl acetate is satisfactory for the separation of oxygenated compounds of slight polarity and even for monophenols.

Other solvent mixtures can also be of value and those whose constituents have different polarities often give the best results. This type of solvent apparently undergoes demixion (caused by "frontal chromatography") which slows down the progress of the more polar constituent; two fronts of elution (in the case of binary mixtures) are thus created separated by a concentration gradient. This may considerably improve certain separations.

Special methods of detection

(*a*) *Detection with* $KMnO_4$. An excess of an aqueous 0.25–0.50% solution of potassium permanganate is placed over the whole surface of the chromatoplate at room temperature. The solvent used in the development must previously be completely removed. Dark spots corresponding to oxidizable compounds appear almost immediately. After about 10 seconds, the plates are immersed in running water to remove the excess of permanganate (properly prepared silicic layers do not disintegrate even on prolonged contact with water). After being washed, the plates are withdrawn and allowed to dry. Very clear brownish spots are obtained on the white background.

(*b*) *Detection with iodine vapour.* Although this method is less convenient than the preceding one, it can be very useful for the examination of compounds which are susceptible to dehydration on heating.

The chromatoplates are heated to 100–150° C and are then immediately immersed in an atmosphere of iodine vapour. After a few seconds, they are withdrawn and reheated to remove the excess of iodine, Brown spots on an off-white background are obtained. The starch in the adsorbent layer does not interfere.

(*c*) *Use of diazotized p-nitraniline for the study and detection of phenols.* The following reagent mixture must be prepared immediately before use: 1 volume of an

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aqueous solution of 0.7 g of *p*-nitraniline and 9 ml of concentrated hydrochloric acid per 100 ml, 1 volume of 1% sodium nitrite solution, and 2 volumes of 5% sodium bicarbonate solution.

The chromatoplates are thoroughly dried, sprayed with the above mixture, and again dried at 40–50° C. The phenols appear as brownish stains.

RAYBURN *et al.*[24] analysed mixtures of phenols by paper chromatography and detected them by means of the colours produced on coupling with diazotized *p*-nitraniline. The writer has adapted this method to chromatoplates in the following way.

Ten μg of each phenol or the mixture under examination in methanolic solution are placed on the starting points of a chromatoplate. The above-mentioned reagent is sprayed on these positions while the rest of the adsorbent surface is protected by a screen. After drying for 10–15 minutes at 40–50° C, the plate is allowed to cool in a desiccator and then developed with a mixture of *n*-hexane and ether (2:1). The dried developed plate is sprayed with a 10% sodium carbonate solution. The initial phenols or types of phenols present can then be identified by the appearance of characteristic colours in conjunction with the R_F-values.

(*d*) *Detection with ferric chloride.* Certain phenols and enols can be detected by treatment with a 5% solution of ferric chloride in a mixture of methanol and water (1:1). If necessary heat at 30–40° C.

(*e*) *Detection of esters.* Volatile esters can be examined directly on chromatoplates by a modification of the hydroxamic acid reaction described by FEIGL[26]. This reaction is not very sensitive on silicic acid but can be transferred to paper in the following manner:

The active layer of the chromatoplate is freed from all organic solvent, moistened by spraying lightly with water and then covered with a sheet of Whatman No. 1 paper of the same size which has been previously soaked with hydroxylamine (see below). A glass plate is placed over the paper to maintain proper contact between the paper and the silicic acid layer and the whole is laid on a warm surface (30–45° C). The esters evaporate together with the moisture from the silicic acid and are fixed in the impregnated paper where they are converted to the hydroxamic acids. After a variable time (10–15 minutes at 30° C for simple benzyl esters), the sheet is removed and sprayed with a solution of 0.5 *N* hydrochloric acid containing 5% of ferric chloride. The characteristic violet spots which appear correspond to the positions of the esters on the original chromatoplate.

As little as 6 μg of benzyl acetate, for example, can readily be detected by this method. On the other hand, esters which are not sufficiently volatile cannot be located. The solvent used for the development of the plates to be examined by this means must of course be free from esters.

Preparation of paper impregnated with hydroxylamine: Just before the paper is required, equal volumes of a solution of 7 g of hydroxylamine hydrochloride in 100 ml of water, and of a solution of 12 g of potassium hydroxide in 100 ml of methanol are mixed. The mixture is sprayed on to the paper which is used immediately while still moist.

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APPENDIX

Since the first publication (in French) of this review (*J. Chromatog.*, 1 (1958) 24), several articles have appeared in the literature, dealing with the use of chromatostrips or chromatoplates. Thus, the following references must be mentioned:

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HIGH VOLTAGE ELECTROPHORESIS

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I. INTRODUCTION

The introduction of paper chromatography at the end of the Second World War led to tremendous advances in the investigation of materials of low molecular weight. However, the method has several disadvantages, such as long working times, uncertainties in the relationship between the R_F-value and the chemical constitution, and, in Central Europe, difficulties in obtaining suitable paper and solvents. This has led to a search for methods which would avoid the drawbacks of paper chromatography whilst preserving similar possibilities of separation.

The technique of paper electrophoresis appears to be very suitable for this purpose; the method was developed by WIELAND AND FISCHER[137,139] but, if electrocapillary analysis is included, its invention is more correctly ascribed to PAUL KÖNIG[65,66]. In this technique, the different velocities of migration of substances in an electric field are utilized for their separation. A strip of filter paper acts as carrier for the conducting solvent and for the sample mixture which is to be resolved into its component parts by means of an applied electrical field. The method is very simple and elegant; given only a source of potential, electrodes, filter paper and two glass plates, amounts of material in the microgram range can be examined. However, with this simple arrangement, each experiment requires several hours or days, for only a potential gradient of 2–10 V/cm can be used; moreover, the sharpness of separation of compounds of low molecular weight is often unsatisfactory. The close interconnection of these properties can be shown by the following simplified example[79]. If two compounds are applied in one spot of diameter 0.5 cm and this spreads during the time of electrophoresis by 0.5 cm to 1 cm, then the compounds must draw at least 1 cm apart to give a detectable separation. If the test period is kept so short that the spot does not spread, then either half the displacement suffices, or with the same displacement, twice as good a separation is obtained. Since an increase in spot size of the above-mentioned extent can readily occur within 2 hours with compounds of low molecular weight[69], an improvement in the sharpness of separation can only be expected if the rate of migration is increased considerably. From these considerations, a drastic increase in the potential gradient must be accompanied by the desired improvement in resolving power.

In electrophoresis, when the potential gradient is increased, the rate of migration rises linearly but the amount of heat generated increases quadratically. It is therefore indispensable in investigations with high potential gradients to remove this heat as far as possible. The apparatus normally used in paper electrophoresis is naturally not

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constructed to fit these requirements, and the expedient of decreasing the buffer concentration, and thus the current intensity and the heat generated, is only applicable in exceptional cases[29,84,109]. The apparatus of BERRAZ[12], which is said to have a certain resemblance to that shown in Figs. 1 and 2[22,78], is completely unsuitable because of its lack of any cooling device. The apparatus of CREMER AND TISELIUS[26] appears to be the best suited to work with high potential gradients; the bands of filter paper are mounted between glass plates and lowered into a trough containing

Fig. 1.

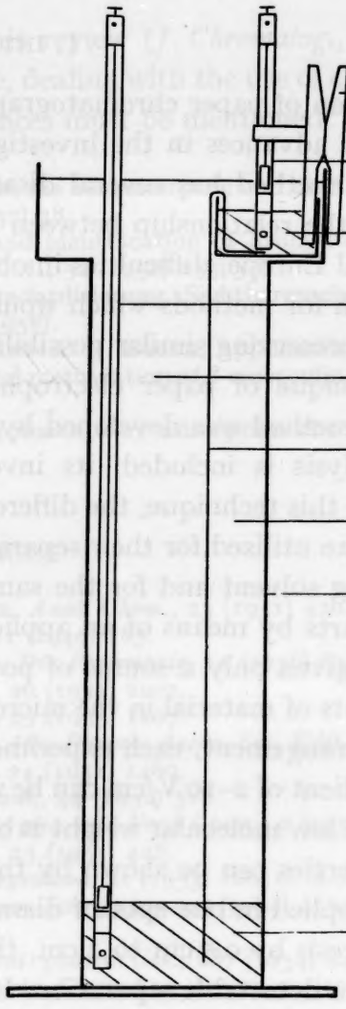

Fig. 2. 1. Filter paper strip. 2. Organic solvent. 3. Glass vessel. 4. Buffer slurried with cellulose paper. 5. Electrodes (Ag/AgCl). 6. Clip.

chlorobenzene. Excellent cooling is thus guaranteed but the diagrams obtained for compounds of low molecular weight are often blurred.

An arrangement was described by the writer[79], which is suitable for this type of work and permits the use of potential gradients up to about 50 V/cm. WESTPHAL *et al.*[136] have coined the name "Hochvoltelektrophorese"—high voltage electrophoresis—for investigations involving potential gradients of this level or higher. Although this designation cannot be defined exactly, it has become widely adopted and will be used here.

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A whole series of different types of apparatus has now been developed. Various cooling arrangements are possible[84] but, at present, only two systems are favoured; these are apparatus with liquid heat exchangers and those with solid heat exchangers.

2. APPARATUS

Apparatus with liquid heat exchangers[25,51,62,68,79,95,102,104,106,115,118,122,125]

Such apparatus can often be assembled from components which are readily available in any laboratory. Despite their simplicity, their resolving power for many classes of compound is the same as that of much more expensive apparatus with solid coolants described below. The disadvantages are the somewhat troublesome manipulation and the fact that the liquids used dissolve many substances.

This apparatus (Figs. 1 and 2) consists essentially of a vertical tube (3) filled with an organic solvent (2); electrode vessels (4, 5) connected to a power supply are placed at the top and bottom of the cylinder. In the apparatus shown, the upper electrode vessel is very simply attached to the wall of the cylinder with a clip (6). The electrode chambers are filled with a slurry of buffer solution and cellulose powder. A strip of filter paper is moistened with the buffer solution and pressed lightly between filter papers, and the sample is applied longitudinally in the form of a streak with a brush or a capillary. The paper is then hung between the electrode chambers, both electrodes (5) are inserted, and the cylinder is filled with toluene or another organic liquid. The current can then be switched on and the experiment started.

If the paper becomes "heated" by the electric current, the warmth is transferred to the organic phase and a convection current is set up. The organic phase first rises and then sinks to the bottom along the cool walls of the vessel. This type of cooling is very effective and its efficiency can easily be further increased by the application of strong external cooling. Up to 0.2 W/cm^2 of the area of the strip can be removed. Apparatus has also been described where the strip is arranged horizontally[25,95,106,118]. Here cooling can be improved by stirring.

The organic phase should fulfil certain requirements; it should be immiscible with the buffer used and with the substance under examination, easily removed from filter paper, and non-inflammable, and it should not have a noxious vapour, etc. Various workers have recommended chlorobenzene[26], hexane or heptane[51], carbon tetrachloride[77] and toluene[79]. Table 1 gives a summary of their physical and chemical properties which are of interest in this connection[4,26,57].

TABLE 1

Compound	*Thermal conductivity in cal · cm^{-1} · degree^{-1} · sec^{-1}*	*Solubility in water (g/100 ml)*	*Vapour pressure in mm Hg at 20° C*	*Temperature of spontaneous ignition*	*Explosion limits in mixture with air*
Chlorobenzene	0.00030	0.049 (30°)	9	—	—
Heptane	0.00034	0.0052 (16°)	35	233°	1–6%
Carbon tetrachloride	0.00028	0.077 (25°)	91	—	—
Toluene	0.00036	0.063 (25°)	22	549°	1.3–7%

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In apparatus with liquid coolants, the manipulation of very large vessels is generally inconvenient so that the length of the filter paper, and thus the distance available for the separation, is usually limited. Therefore it has been proposed that, instead of the strips being hung directly between the electrodes, they should be held at an angle[122,125], supported in a zig-zag shape[95,106], or wound in rolls[8,145].

As mentioned already, the filter paper strips should be pressed between filter papers and should be relatively dry before they are placed in position (weight of air-dried strip:buffer should be approximately 1:1.2). To ensure that the excess buffer can be absorbed only slowly from the electrode vessels, these are filled with a slurry of filter paper and buffer[81,122]. Unless this precaution is taken, there is a danger that the applied substance may be washed away completely ("wick effect").

In investigations which take some time, it is also expedient to use reversible electrodes, *e.g.* as in Figs. 1 and 2, two silver/silver chloride electrodes separated by a diaphragm from the electrode chambers.

Apparatus with solid heat exchangers[10,27,32,43,82,90,133,134,140]

In this second type of apparatus, the heat is removed by cooled, electrically insulated plates. The strips of filter paper can be laid between two such surfaces[43,82], but it is often sufficient to use only one and to cover the strips with a plate of some transparent material. In Figs. 3 and 4 is shown an apparatus of this type[87,90] which has been used in this Institute for the past 6 years without essential alteration or repair. It consists essentially of a condenser (3) covered with a glass plate (2). The buffer vessels (5) are situated at the ends and are connected with the reversible electrodes (8) through a bridge (6). All these components are housed in a plastic casing (9).

At the beginning of the investigation, the half-moist paper strip (1) is laid on the glass plate (2) and the substance is applied. The paper strip (1) and the buffer vessels (5) are connected by means of a piece of cellophane tubing (4) which is moistened with buffer and provided with a wick of filter paper. After the strip has been covered with a glass plate (2), the electrode vessels and the bridges, filled with buffer, are inserted, the entire case is covered with a lid (not shown), and the source of potential is connected.

The moisture content of the strip is of some importance[82,86,130]. From the viewpoint of the best possible removal of heat, it is of course preferable to have the strip so wet that the buffer solution is in direct contact with the cooling surface; in this case the ratio of the weight of the air-dried strip to that of the buffer is 1:2.0–2.5. However, the resolving power of the method is greatly diminished by such a procedure, for the buffer and the substance solution run off the paper much more readily. Moreover, different conditions of migration prevail in the filter paper and in the film of buffer between the paper strip and the cooling surface. If, as stipulated above, a relatively dry strip is inserted (weight of air-dried strip:buffer is 1:1.0–1.2)[82], then the strip is kept at a distance of some hundredths of millimetres from the cooling surface by means of the cellulose fibres. We have also used a very narrow "wet chamber" similar to that employed in the larger scale methods of low voltage electrophoresis.

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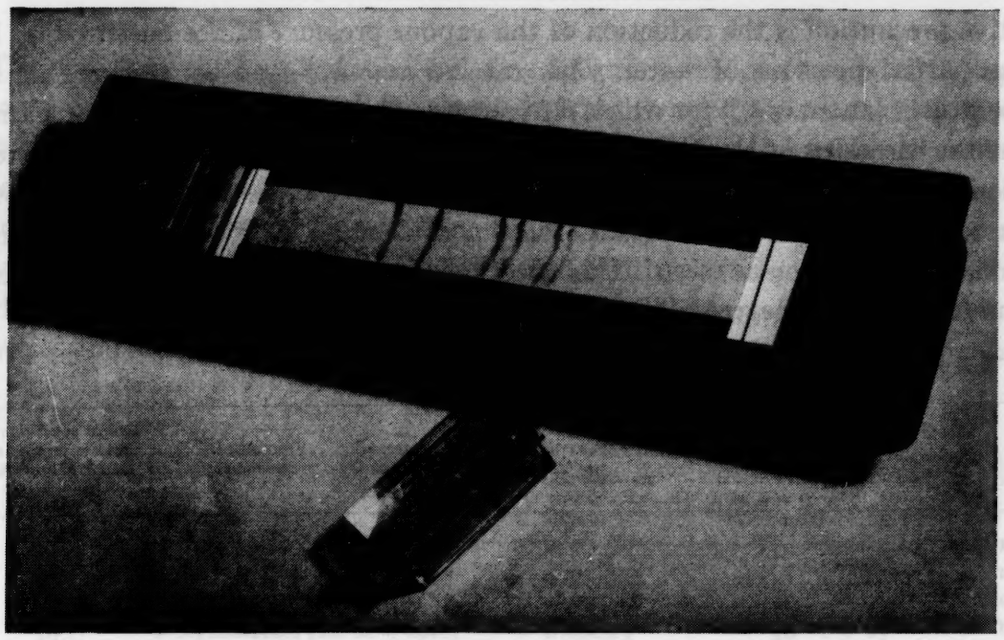

Fig. 3.

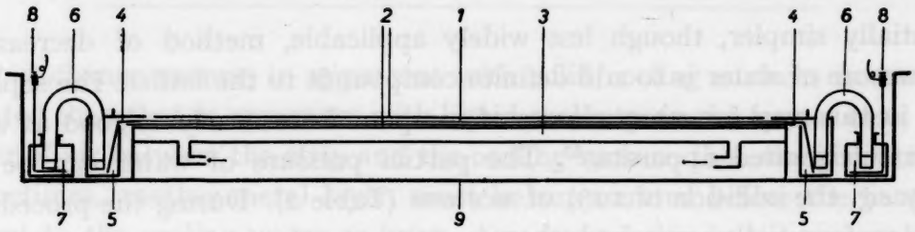

Fig. 4. 1. Filter paper strip. 2. Glass plates. 3. Condenser. 4. Cellophane membrane. 5. Buffer container. 6. Bridge. 7. Electrode chamber. 8. Electrode ($Cu/CuCl_2$). 9. Case.

In order to prepare reproducibly strips of the desired content of moisture, they are first drawn through the buffer solution, squeezed out between filter papers or by means of a sort of washing wringer[133,134], and then their weight is checked. A half-dry strip prepared in this way tends to absorb fresh buffer from the buffer vessels after being placed in the apparatus, hence it should be isolated from the flow of liquid by means of a cellophane membrane. FOSTER[32] avoids the necessity of this procedure in his apparatus by allowing the migrating substance to travel in the electric field against the buffer current; thus the mobilities found in his apparatus ([37], *cf.* Table 10, p. 26) are often much smaller than those found by other methods[82].

The greatest possible part of the heat developed in the strip by the electric current should be removed by conduction of heat rather than as heat of vaporization; otherwise the buffer solvent distils to the cooling surface and there forms large drops. When these drops are again absorbed by the strip, the substance is washed away from these positions and, after development, the strip adopts a characteristic mottled appearance (*cf.* Table 5). If the cooling surfaces are as hydrophobic as possible (glass covered with silicone grease or vaseline, PVC-plates, etc.), the occurrence of supersaturation phenomena is promoted and condensation is thus avoided. Another very

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effective precaution is the reduction of the vapour pressure of the buffer or, at least, of the partial pressure of water. This can be accomplished by working at lower temperatures (about 0–5°) for which refrigeration is necessary[133,134]. At these temperatures the viscosity of the buffer increases greatly (see Table 2), and the potential gradient must be raised correspondingly in order to overcome the effect. As shown in Table 2, the potential gradient must be almost doubled at 0° in order to attain the same rate of migration as at 20°. This effect is intensified under certain circumstances by the fact that the degree of dissociation is also dependent on temperature.

TABLE 2[4,57,67]

	Temperature in °C	*Partial pressure of water in mm Hg*	*Viscosity in centipoise*	*Potential gradients giving same migration rates (calculated from Stokes' law) V/cm*
Water	0	4.58	1.78	89
Water	10	9.20	1.30	65
Water	20	17.53	1.00	50
Water with 10 % (w/w) acetone	20	8.0	1.18	59
Water with 10 % (w/w) glycerol	20	17.0	1.31	65

An essentially simpler, though less widely applicable, method of decreasing the partial pressure of water is to add definite compounds to the buffer. The separations obtained in this way for many classes of compound are nearly as good as with the expensive refrigerated apparatus[87]. The partial pressure of water can be almost halved by *e.g.* the addition of 10% of acetone (Table 2). During the procedure, the acetone is preferentially vaporized, then condenses on the cooling surface and is again absorbed by the paper. However, since the vapour phase in contact with the specified mixture contains approximately 80% of acetone and most of the substances investigated do not dissolve in acetone, no interference occurs[87].

With buffers which contain a relatively large amount of slightly dissociated compounds, *e.g.* the pyridine–acetic acid buffer[79], this effect is inherently present. Glycerol and similar compounds are not suitable additions in the present case, for they increase the viscosity of the buffer too much without lowering the partial pressure of water correspondingly.

The potential gradient and the current intensity have an upper limit due to the restricted capacity of the apparatus for removing the warmth evolved. The material from which the cooling surface is made is extremely important for the heat exchange. The requirements for this material are very contradictory: on the one hand, it must be an electrical insulator and possess high dielectric strength, and on the other hand, it must be a good heat conductor. According to the Widemann-Franz rule, both these conductivities run in parallel. Glass and ceramic materials (Table 3) have the most favourable properties of the usual commercial materials. They are also much more resistant to organic liquids than *e.g.* plastics for work with non-aqueous solvents[94]. Nevertheless, apparatus constructed completely from PVC or polymethacrylic acid esters has the advantages of lesser fragility and greater ease of manufacture.

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The necessary thickness of the insulating layer depends on the size of the apparatus and thus on the total voltage applied. Smaller apparatus permit only short separation distances, but thinner insulating layers which are better heat conductors can be used[82] and the potential gradients can accordingly be increased. In this way, amounts of heat corresponding to an output of several tenths Watt/cm² can be removed. Most other apparatus, especially those with plastic plates, should not be loaded with more than 0.05–0.1 Watt/cm². In Table 3 are given the thermal conductivities and dielectric strengths of several materials used in high voltage apparatus.

TABLE 3[4,103]

	Thermal conductivity in cal·cm^{-1}·sec^{-1}·degree^{-1}	*Dielectric strength in kV/mm (from VDE 0303)*
Air	0.000062	*ca.* 1
Polyvinylchloride (PVC)	0.00039	50
Polymethacrylic acid ester	0.00043	40
Glass	*ca.* 0.002	20–40
Aluminium	0.585	—

The heat exchange process in apparatus with solid cooling surfaces is much more complicated than that in apparatus with liquid coolants. In the former case, first the thin layer of air between the strip and the cooling surface, then the insulating layer and, sometimes, another metal layer, must be surmounted before the heat can be transferred to the cooling water or brine. Any irregularity in the removal of heat causes distortion in the zones, for a difference in temperature of 1° produces a difference of about 3% in the rate of migration[26]. The paper strips must be placed on, and fixed to, the cooling surface very carefully. Generally it is sufficient to use a plane polished glass plate (plate glass); in several apparatus, a special stretching arrangement is provided[133,134].

Apparatus for special purposes

(*a*) *For large or small amounts of material.* The methods and apparatus outlined above permit separations of amounts of material of the order of 10^{-6} to 10^{-3} g. As is briefly mentioned in the section on proteins, larger amounts can be separated by HVE in thin plates of *e.g.* starch[84,140] or by free zone electrophoresis[85]. Special apparatus is required for the investigation of smaller amounts of material of the order of 10^{-7} to 10^{-9} g (see *e.g.* [31,93,127]). Soaked threads or thin liquid films can be used as carriers for the buffer; since these can be cooled very easily, very high potential gradients can be applied[87].

(*b*) *For electrophoresis and chromatography.* The resolving power for any separation can be greatly increased by a combination of high voltage electrophoresis and chromatography. Generally, the substances are isolated after one technique has been used and then reapplied. This is very laborious and it is simpler to prepare a two-dimensional diagram; electrophoresis is used in one direction, preferably with a volatile buffer, and then the paper is dried and developed in the second direction by paper chromatographic

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techniques[56,62]. Of course, electrophoresis at a different pH-value can also be used for the separation in the second direction; this was shown by DURRUM[29] some time ago.

(*c*) *For isoelectric separation.* Compounds are generally separated by means of their different electrophoretic mobilities in a paper strip but they can also be separated by means of their different isoelectric ranges or, commonly, by the dependence of their mobilities on pH (*e.g.* [110]). For this purpose, it is necessary to create a pH-gradient in the direction of the electric field and to keep this gradient temporarily constant. The former can be accomplished relatively easily by diffusion of a second buffer of different pH, or by electrolysis in a semiconductor, etc.; the temporary constancy is difficult to achieve and this is probably the reason why the resolving power of normal high voltage electrophoresis cannot be attained, at least not for compounds of low molecular weight. However, a number of investigations which are not cited here have been carried out.

3. BUFFERS AND SOLVENTS FOR HIGH VOLTAGE ELECTROPHORESIS (HVE)

The selection of the correct solvents and buffers is just as important for satisfactory separations as the apparatus itself. It is not always sufficient for the pH-value of the buffer to lie between the pK-values of the substances under investigation[23] or, in the case of complex formation, the analogous function of complexant concentration; adsorption and displacement phenomena must also be considered. This is easiest in the case of adsorption phenomena. If the material under examination is chromatographed on paper with the buffer which is to be used in the electrophoretic method, and if tailing and R_F-values of less than 1 are obtained, then tailing towards the position of application and slow migration are to be expected with electrophoresis. This effect is especially marked in the case of basic organic substances such as alkaloids and dyestuffs. The apparently paradoxical properties of arginine, which often migrates more slowly than lysine in paper ionophoresis despite its higher isoelectric point, are also caused by this effect. Although separations can sometimes be obtained directly owing to such adsorption phenomena, the effects are usually very undesirable. In the case of true adsorption on the cellulose fibres, it is difficult to find a remedy; often, the only satisfactory method is to use a non-aqueous solvent, or one of low water content, or to substitute plastic paper, *e.g.* Schleicher and Schüll's "Rhovylpapier" 1001 which consists of PVC-fibres[87,116]. In the latter case, a certain amount of distortion of the bands must be accepted because of the uneven texture of the paper. If the adsorption is limited to ion exchange (free carboxyl groups in the cellulose), it is often sufficient to use buffers containing alkaline earth metal ions[80,81,87]. Finally, displacement phenomena often occur, especially with inorganic ions, but these tend to promote the separation.

Substances of low molecular weight are not adsorbed generally form "shadows" in the direction of migration; this is particularly evident when the paper is overloaded with the test material. Thus the compound migrates more slowly at the positions where higher concentrations are present than it does where smaller concentrations occur, because the field strength is reduced by the greater conductivity. The compound migrates more quickly at the front and rear of the zone where the concentrations are

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smaller. At the front, it migrates into the buffer, distributes itself over a greater area and makes the boundary between the compound zone and the buffer indistinct; whereas at the rear it is drawn further into the zone itself so that the boundary remains sharp. The analogous phenomena for free electrophoresis are well-known and have been treated mathematically[119,129,131].

A multiple solvent with volatile constituents is more convenient than a buffer containing a salt, because it can be removed from the strip simply by volatilization or sublimation. Solutions which have proved particularly useful are mixtures of formic and acetic acids (pH *ca.* 2)[62], pyridine–acetic acid (pH 3.5–6)[51,79,102], ammonia–formic acid or acetic acid (pH 6–10)[54,88], trimethylamine–formic acid, acetic acid and carbonic acid (pH 3–6 and 7–12)[96], collidine–acetic acid (pH *ca.* 7)[92], ammonium carbonate (pH *ca.* 8)[24]. Less experience has been gained as yet with collidine–hydrochloric acid (pH 6.5–8.3)[99], mono- or triethanolamine–hydrochloric acid (pH 6.5–11)[120] and similar mixtures.

As mentioned above, it is in many cases preferable to substitute another liquid for water as the main constituent of the solvent. Interfering adsorption phenomena are often decreased in such systems and, moreover, compounds which are insoluble in water can be examined. The disadvantages are the small conductivity of these solvents and the fact that the filter paper does not expand in them. Moreover, only apparatus containing glass cooling surfaces can be used in this type of experiment.

DURRUM AND PAUL[30] showed, for example, that a number of organic dyestuffs which were strongly adsorbed in aqueous buffers migrated satisfactorily in high-percentage alcohol. The dyestuff crystal violet is firmly retained by swollen cellulose fibres and therefore does not migrate in normal paper electrophoretic tests, whereas it moves very clearly in various organic solvents (Table 4).

TABLE 4[94]

Solvent	*Dielectric constant*	*Viscosity in centipoise (20° C)*	*Distance of migration in cm at 90 V/cm during 20 min*
Ethyleneglycol	41.2	19.9	0.7
Nitromethane	39.4	0.66	3.8
Dimethylformamide	36.7	3.76	6.0
Nitrobenzene	35.7	2.01	3.2
Ethanol	28.8	1.19	4.2
Isopropanol	26.0	2.39	5.0
n-Butanol	19.2	2.95	2.5
Pyridine	13.6	0.95	4.0
Ethylenedichloride	10.5	0.83	0.7

When solvents are being chosen, it would be expected from Coehn's rule and Stokes' law that those of higher dielectric constant and lower viscosity would be most suitable but here again there are exceptions, as can be seen from the example of dimethylformamide in Table 4.

The dissociation of a compound in an organic solvent is usually smaller than in water, hence the substance under investigation must be sufficiently easily convertible

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to an ionized state. If the compound cannot itself be ionized, the introduction of a suitable functional group is often successful. For example, HASHIMOTO[49] converted triterpenes to their sulphonic acids by treatment with sulphur trioxide, and then separated them in butanol–acetic acid mixture.

Recently, fused inorganic salts have been used as the electrolyte with asbestos paper as the carrier[21]. Isotopes can be separated successfully in such systems; this is fully described by one of the authors in another part of this volume (see pp. 246–267).

TABLE 5

SOME ERRORS OCCURRING IN HIGH VOLTAGE ELECTROPHORESIS AND THEIR REMOVAL[87]

Symptom	*Cause*	*Remedy*
Substance zones are clearly defined but deformed	Uneven heating, ionic strength, pH or moisture content; uneven paper texture; uneven drying	Strip should be in even contact with cooling surface throughout, uniformly moist, or equilibrium should be awaited after application; desalting may be needed
Zones form crescents	Cooling at strip edges is stronger than in centre	Apply strips on the sides[88,133,134]
Zones seem mottled and blurred	Cooling is insufficient, cooling surface too wet	Decrease heat development, keep cooling surface dry and hydrophobic, perhaps apply vertical heat gradients[132]
Abnormal migration rates	Wick effect[81]. Current stopped. Electroosmosis (esp. with high pH-values and in glass paper). Buffer congealed	Check cellophane barriers and current strength, add non-migrating substance to detect starting point
Paper dry and smouldering on sharply defined bands, or transparent with liquid heat exchangers	Paper is badly uncovered (esp. near buffer vessels), different buffers in strip and buffer vessels, pH-shift, coolant not saturated with buffer	Obvious from the cause
Tailing *in* the migration direction	Excessive concn. of test substance; rarely, pH-effect[22]	Obvious from the cause. Increase ionic strength of buffer
Tailing in *opposite* direction to migration	Adsorption; rarely, pH-effect[22]	Work at lowest possible pH-values, add organic solvent to buffer, use buffer with organic base or alkaline earth ions, use glass-fibre or plastic paper[11,87,116]
Test substance forms bands back to start	Substance incompletely dissolved; paper scratched[140]	Obvious from the cause
Shrinkage of the substance zone	Substance was applied with another solvent (*e.g.* from alcohol)[140]	Obvious from the cause

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4. CLASSES OF COMPOUND INVESTIGATED

Amino acids

Mixtures of amino acids have frequently been examined by means of high voltage electrophoresis[1,2,45,50,51,62,79,89,102,115,133,134,140,141]. Moreover, a new amino acid (δ-oxyleucenine) has been detected by HVE in the hydrolysate of amanita poison[142].

The analysis of a mixture of natural amino acids, *e.g.* protein hydrolysates, is most successful at a pH-value of about 2[45,62,89,115,133,134]. As is shown in Fig. 5, the separation

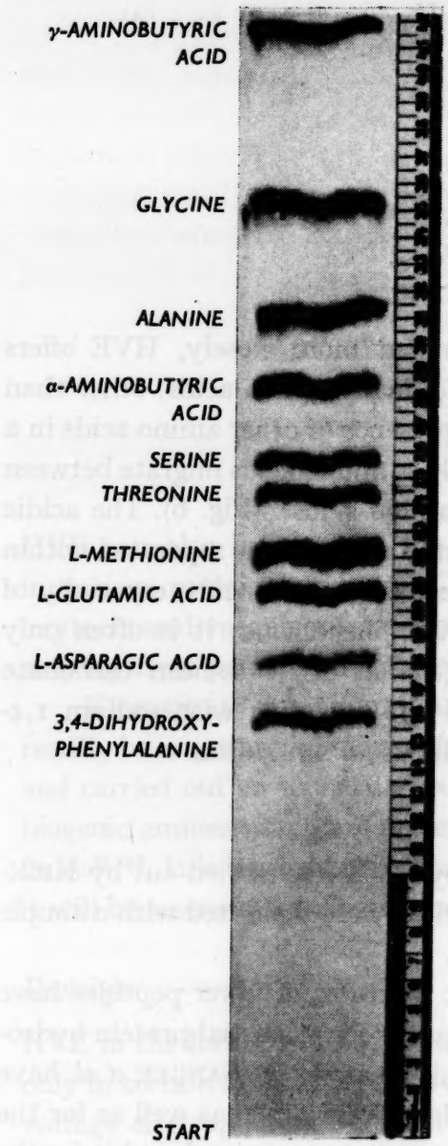

Fig. 5. Apparatus as in Fig. 3; 40 V/cm, 2 mA/cm of the width of the strip; 120 min; 10°. Solvent: 3 ml 80% formic acid, 12 ml glacial acetic acid, 15 ml acetone diluted to 100 ml with water (pH 1.9). Colour reaction: 0.2% ninhydrin in butanol.

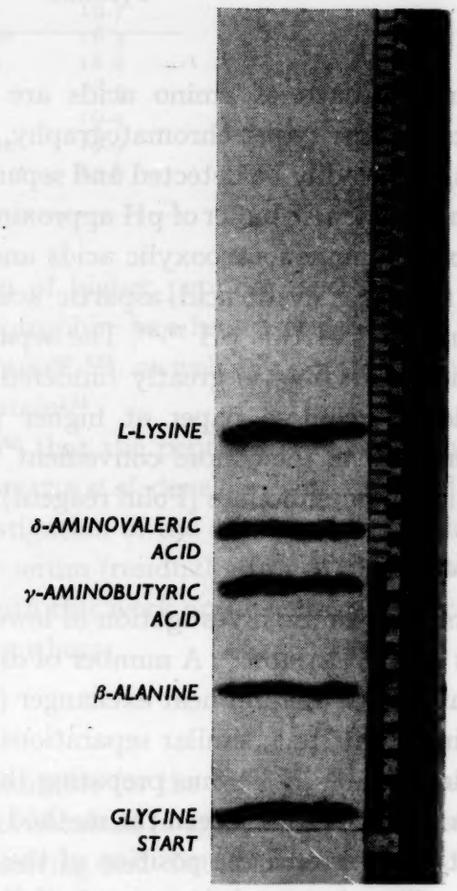

Fig. 6. Apparatus as in Fig. 3; 33.5 V/cm, 3.7 mA/cm of the width of the strip; 90 min; 10°. Solvent: 2 ml pyridine, 4 ml glacial acetic acid, 15 ml acetone diluted to 100 ml with water (pH 4.4). Colour reaction: 0.2% ninhydrin in butanol.

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obtained is similar to that obtained on a good unidimensional paper chromatogram. Some figures for the migrations rates are given in Table 6.

TABLE 6

RATIO OF THE MIGRATION DISTANCES OF AMINO ACIDS/ALANINE[141] TOWARDS THE CATHODE

Formic acid–acetic acid mixture[62] of pH 1.9

Amino acid	*Ratio*
Lysine	1.4
Histidine	1.29
Arginine	1.27
Glycine	1.15
Alanine	1.00
Serine	0.84
Valine	0.82
Leucine	0.77
Isoleucine	0.77
Methionine	0.72
Glutamic acid	0.69
Aspartic acid	0.64
Tyrosine	0.55

If definite groups of amino acids are to be examined more closely, HVE offers advantages over paper chromatography. For example, many amino acids, other than α-acids, can readily be detected and separated in the presence of other amino acids in a pyridine–acetic acid buffer of pH approximately 4.5. These amino acids migrate between the monoaminomonocarboxylic acids and the basic amino acids[89] (Fig. 6). The acidic amino acids, *e.g.* cystic acid, aspartic acid and glutamic acid, can be separated within a few minutes at this pH [79,89]. The separation of the basic amino acids, especially of arginine and lysine, is greatly hindered by adsorption phenomena; it is often only possible on cellulose paper at higher pH values (*e.g.* in 0.1 *N* sodium carbonate solution)[87]. It is then more convenient to carry out the detection with sodium 1,4-naphthoquinonesulfonate (Folin reagent) instead of the usual ninhydrin.

Peptides

The first systematic investigation of lower peptides by HVE was carried out by KICKHÖFEN AND WESTPHAL[63]. A number of di- and tripeptides were separated with a simple apparatus with a liquid heat exchanger (see Table 7).

Since that time, similar separations of synthetic mixtures of lower peptides have been described[45,133,134] thus preparing the way for separations of partial protein hydrolysates. HVE has often been the method chosen for this purpose[1,108]. SANGER *et al.* have used it to determine the position of the sulphur bridges in insulin[102] as well as for the examination of pig and sheep insulin[18,50]. REDFIELD AND ANFINSEN employed it for the analysis of peptide mixtures obtained by the enzymic decomposition of ribonuclease[100,101]. These authors also described the separation of DNP- peptides by HVE. In order to overcome the adsorption phenomena which occur with these compounds and to increase the solubility, they recommended the addition of a considerable amount of

References p. 35.

urea to the buffer. However, such an addition must be used with caution for the commoner peptides, because its desorbing action is then slight and it interferes with the ninhydrin reaction. TURBA AND ESSER[123] followed the rate of introduction of ^{14}C into amino acids, peptides and proteins by adding labelled acetate to Torula yeast; the very complex mixture resulting from this treatment was separated by means of HVE and paper chromatography.

TABLE 7

SEPARATION OF PEPTIDES

Migration to the cathode in cm, 70 V/cm, 1.1 mA/cm of strip width. Formic acid–acetic acid mixture of pH 1.9, 60 min

Peptide	*Migration in cm*
Alanylglycine	22.8
Glutathione	5.6
Glutaminylglycine	18.3
Glycine	16.4
Glycylglycine	23.9
Glycylglycylglycine	21.4
Glycylleucine	19.7
Glycylleucylalanine	18.3
Glycylvalylalanine	18.8
Glycyltyrosine	16.5
Leucylglycine	19.4
Leucylglycylglycine	17.9
Leucyltyrosine	15.6

HVE can also be used for the investigation of higher peptides, such as are present in various protein hormones. Choriongonadotrophin has been isolated from urine[91], oxytocin and vasopressin from pituitary extracts[87,121], as well as a peptide of oxytocic activity from partially hydrolysed serum protein[124].

KICKHÖFEN AND WESTPHAL had shown[63] that the peptides found in urine could readily be examined by means of HVE. HEILMEYER *et al.* developed their method further and carried out an unusually extensive investigation of the amino acids, peptides and biogenic amines which are present in human serum (residual nitrogen) and other body fluids[51,52]. It is impossible to deal properly with this work and its clinical aspects here; it will be reserved for a monograph by these authors.

Proteins

HVE in the form described here is not very suitable for the separation of proteins; for only in certain favourable cases are the results obtained in agreement with those of low voltage electrophoresis[81,109,118]. Generally, blurring and distortion of the bands occur particularly on half-dry strips, hence good separations are only to be expected in cellulose paper when the paper is in a very wet condition (weight of dry paper: buffer is approximately 1:2–2.5) and when gaseous heat exchangers are used. Of course in this case it is not easy to obtain adequate cooling. Various possibilities have been examined (*e.g.* transference of the heat to a gas, such as hydrogen or helium, which is a good conductor[78],

References p. 35.

transference by radiation[84], suspension in a vertical cloud chamber[55,84]), but it is still simplest to cool by evaporation of the buffer or its solvent. The wick effect[97,98] which then occurs can be utilized to improve the separation by suitable shaping (trapeze- or circular shape) of the paper[13,84]. Potential gradients of approximately 20 V/cm can thus be utilized.

In general, it is also possible to discard the use of cellulose paper and to adopt other materials or modes of procedure for the stabilization of the buffer. Among the different possibilities are, for example, plastic paper[116], starch or similar hydrophilic powders[84,139], gels of starch or similar substances[113], and electrophoresis chambers of suitable shape[85]. In all of these cases, potential gradients of 20–50 V/cm can be used so long as efficient cooling is provided (apparatus with solid heat exchangers) and the experimental set-up is kept as flat as possible[87]. Particular experience has been gained in the use of thin layers of starch, and mixtures of the more sensitive enzymes can be extensively broken down and examined (*e.g.*[83,141]). Electrophoresis as introduced by SMITHIES in concentrated starch gels has also proved very effective[113]. With these gels, fractionation according to the particle size by a sort of ultrafiltration occurs in addition to the electrophoretic separation. Under these conditions, human[111,113,114] and animal sera[5] can be fractionated further than by any other electrophoretic method.

Biogenic amines

In electrophoresis patterns of biological materials, bands which are only delicately coloured by ninhydrin, are often found between the basic amino acids and the cathode. These are generally caused by biogenic amines.

Their occurrence in urine has been thoroughly examined by WEBER[130] and later by HONEGGER[56]. Ammonia, methylamine, dimethylamine, ethylamine, colamine, pyrrolidine and piperidine are among the amines which have been separated by paper ionophoresis in a citrate buffer of pH 3.8 and by subsequent paper chromatography[56]. A number of the amines detected by HEILMEYER *et al.*[51,52] in deproteinized serum (residual nitrogen) were identified by GAYER[42]. These amines were precipitated from the very complex mixtures by means of potassium tetraphenylboron (Kalignost, tetragnost); the precipitate was then decomposed with hydrochloric acid, separated from excess of

TABLE 8[42]

RATIO OF MIGRATION DISTANCES OF AMINES/ARGININE

Pyridine–acetic acid–water–80% formic acid (1:10:89:13.5); pH 2.3; cooling with heptane; about 60 V/cm

Amine	*Ratio*	*Colour with ninhydrin*
5-Hydroxytryptamine	0.65	blue-grey
Adrenaline	0.75	—
Tryptamine	0.87	blue-violet
Tyramine	0.97	pale violet
Arginine	1.00	blue-violet
Histamine	1.66	violet

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reagent by shaking with ether, and, after concentration, examined by means of HVE. Information on the mobility of some of these compounds is given in Table 8. This method is also suitable for the detection of biogenic amines in animal poisons[87].

A number of guanidine derivatives can also be separated very well by HVE and then detected specifically on the paper. Some reference points for the migration rates which can be expected are given in Table 9[73].

TABLE 9

SEPARATION OF GUANIDINE DERIVATIVES

Migration to the cathode in cm; 15 V/cm; 0.4 mA/cm strip width; veronal buffer pH 8.6; $\mu = 0.1$; 120 min; 10°C; colour test with α-naphthol–diacetyl reagent

Guanidine derivative	*Migration in cm*
Citrulline	3.2*
Glycocyamine	3.85
Creatine	4.00
γ-Guanidinobutyric acid	4.00
Arginine	8.05
Arcaine	10.70
Dimethylguanidine	12.85
Agmatine	13.00
Methylguanidine	13.90
Guanidine	14.90

* Detected with Ehrlich reagent.

Carbohydrates and polyvalent alcohols

It has long been known that carbohydrates and polyvalent alcohols form complexes with boric acid (*cf.* the reviews by BÖESEKEN[15] and ZITTLE[146]), but it was not until the end of 1952 that this property began to be utilized for the separation of mixtures of carbohydrates by electrophoretic methods[25,32,48,59,82]. Thus the writer has shown[82] that a number of sugars, polyvalent alcohols and phenols can be separated by HVE in a 0.08 *M* borax solution and that a relationship between migration rates and structure can be discerned. These experiments were later confirmed and extended. WERNER AND WESTPHAL[133,134,135] examined carbohydrates which are present in pyrogenes. GROSS[43,44] occupied himself with disaccharides and a new trisaccharide, kestose; he also separated a mixture of glycol, glycerol, erythritol, glucitol, mannitol and galactitol in a 0.05 *M* borax solution[46].

Quantitative estimates of the mobilities of carbohydrates and polyvalent alcohols have been made repeatedly[25,48,82,117]. However, it is impossible to compare these data directly because of the diversity of the experimental methods involved, especially with regard to the buffer content of the filter paper and the wick effect. FOSTER[32–39] has given by far the most extensive information and Table 10 has been compiled from his data.

The relationship between the disposition of the hydroxyl groups and the mobility of the borate complexes has also been considered[6,34,82]. The hydroxyl groups in the *cis*-positions on the C_1 and C_2 atoms of the sugar are the most effective; this was already

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TABLE 10

RATIO OF DISTANCES OF MIGRATION OF COMPOUND/GLUCOSE (M_G-VALUES)

Apparatus according to FOSTER[32]; 15–22 V/cm[34,9]; borate buffer, pH *ca.* 10 (7.44 g boric acid in 1 l *N*/10 sodium hydroxide, erroneously described by the author as 0.2 *M*)[37]

Carbohydrate	*Ratio*	*Reference*
Amylopectin	0.25*	37
Amylose	0.18**	37
1,4-Anhydro-D-galactitol	0.47	35
1,5-Anhydro-2-deoxyglucitol	0.20	39
1,6-Anhydro-β-D-glucopyranose	0.00	39
2,5-Anhydro-D-mannitol	0.04	9
1,5-Anhydro-D-xylitol	0.00	39
L-Arabinose	0.96	17
D-Arabitol	0.90	17
Ethyl-2-deoxy-α-glucopyranoside	0.17	39
Ethyl-2,3-dideoxy-α-D-glucopyranoside	0.10	39
Ethyl-α-D-glucofuranoside	0.70	39
Ethyl-β-D-glucofuranoside	0.65	39
Ethyl-α-D-glucopyranoside	0.17	39
Cellobiose	0.28	34
1-Deoxy-D-arabinose	0.41	36
1-Deoxy-D-glucose	0.20	36
2-Deoxy-D-ribose	0.33	17
1,3-Dihydroxyacetone	0.78	17
2,3-Dimethyl-D-glucose	0.12	34
2,4-Dimethyl-D-glucose	0.05	34
3,4-Dimethyl-D-glucose	0.31	34
2,3-Dimethyl-L-rhamnose	0.023	32
2,4-Dimethyl-L-rhamnose	0.046	32
3,4-Dimethyl-L-rhamnose	0.36	32
Dulcitol (Galactitol)	0.98	17
meso-Erythritol	0.75	17
D-Fructose	0.90	17
L-Fucose	0.89	17
D-Galactose	0.93	17
L-Galaheptulose	0.89	17
Gentiobiose	0.75	34
	0.72	17
D-Glucose	1.00***	9, 17, 32–35, 37, 39
D-Glucose-1-phosphate	1.10	17
D-Glucuronic acid	1.20	17
DL-Glyceraldehyde	0.79	17
Glycerol	0.44	17
D-Glycero-D-galaheptitol	1.00	17
D-Glycero-D-mannoheptitol	0.92	6
Glycogen	0.31§	37
Lactose	0.38	34
Laminaribiose (3-(β-Glucosido)-glucose)	0.69	34
D-Lyxose	0.67	91
Maltose	0.36§§	37
iso-Maltose	0.69	34
D-Mannitol	0.90	17
D-Mannoheptulose	0.87	17
D-Mannose	0.72	17
Melezitose	0.22	17

* Apparent mobility (def. by [130]) 0.51–69·10^{-6} cm^2 V^{-1} sec^{-1} (Ref. [37]).
** Apparent mobility (def. by [130]) 0.35–57·10^{-6} cm^2 V^{-1} sec^{-1} (Ref. [37]).
*** Apparent mobility (def. by [130]) 2.26–2.74·10^{-6} cm^2 V^{-1} sec^{-1} (Ref. [37]).
§ Apparent mobility (def. by [130]) 0.61–0.90·10^{-6} cm^2 V^{-1} sec^{-1} (Ref. [37]).
§§ Apparent mobility (def. by [130]) 0.81–1.08·10^{-6} cm^2 V^{-1} sec^{-1} (Ref. [37]).

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TABLE 10 (continued)

Carbohydrate	*Ratio*	*Reference*
Melibiose	0.80	34
Methyl-α-D-arabofuranoside	0.035	9
Methyl-α-L-arabofuranoside	0.035	9
Methyl-β-D-arabofuranoside	0.035	9
Methyl-α-D-arabopyranoside	0.40	36
Methyl-β-D-arabopyranoside	0.40	36
Methyl-α-D-fructofuranoside	0.60	35
Methyl-β-D-fructofuranoside	0.04	35
Methyl-α-D-fructopyranoside	0.71	35
Methyl-β-D-fructopyranoside	0.59	35
Methyl-α-D-galactofuranoside	0.41	35
Methyl-β-D-galactofuranoside	0.31	35
4-Methyl-D-galactose	0.27	34
Methyl-α-D-glucofuranoside	0.73	35, 39
Methyl-α-D-glucopyranoside	0.09	34
	0.11	39
Methyl-β-D-glucopyranoside	0.19	39
2-Methyl-D-glucose	0.23	34
3-Methyl-D-glucose	0.82	34
4-Methyl-D-glucose	0.24	34
6-Methyl-D-glucose	0.82	34
Methyl-β-D-gulopyranoside	0.72	35, 37
Methyl-α-D-lyxopyranoside	0.45	36
Methyl-β-D-lyxopyranoside	0.26	36
Methyl-α-D-mannopyranoside	0.42	35
Methyl-β-D-mannopyranoside	0.31	36
Methyl-(4-O-methyl)-β-D-glucopyranoside	0.00	39
Methyl-α-D-xylofuranoside	0.30	39
Methyl-β-D-xylofuranoside	0.30	39
Methyl-α-D-xylopyranoside	0.00	39
Methyl-β-D-xylopyranoside	0.00	39
myo-Inosite	0.53	17
Nigerose	0.69	17
1,2-O-Isopropylidene-α-D-glucofuranose	0.73	9, 35, 39
Raffinose	0.28	17
L-Rhamnose	0.52	17
D-Ribose	0.77	17
Saccharose	0.18	17
	0.17	39
α-Schardinger Dextrin	0.14	37
β-Schardinger Dextrin	0.12	37
Sophorose (2-(β-Glucosido)-D-glucose)	0.33	17
Sorbitol (Glucitol)	0.89	17
α,α-Trehalose	0.19	17
α,β-Trehalose	0.23	39
β,β-Trehalose	0.20	39
2,3,4-Trimethyl-D-glucose	0.00	34
2,3,6-Trimethyl-D-glucose	0.00	34
3,5,6-Trimethyl-D-glucose	0.71	34
D-Xylose	1.00	17

known from conductivity measurements[15,146]. If the glucosidic hydroxyl group is blocked, possibly with a methyl group, this glucoside generally possesses a much smaller mobility than the free sugar. Adjacent alcoholic hydroxyl groups in the ring are also effective, as is shown by the appreciable mobility of methylgalactoside. In some cases (methylxyloside), it is necessary to take into consideration complex formation at

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hydroxyl groups other than those at the *cis*-positions, or the participation of aldehyde structures as in the case of 2-O-methyl-D-glucose[34].

Another means of making carbohydrates migrate in the electric field is to substitute the carbonyl group suitably; this can be achieved by the use of a 0.4 *M* sodium bisulphite solution as buffer[41], or by conversion to the N-benzylglucosylammonium compound[6]. Especially in the former case, there is a definite relationship between the rate of migration and the particle size[41].

Migration also occurs with several polyvalent alcohols when buffers containing lead acetate or sodium arsenite are used[40].

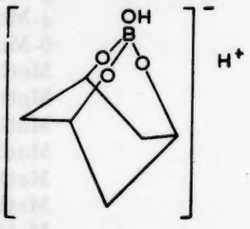
Cyclic alcohols

Numerous polyvalent cyclic alcohols migrate with different migration rates in borate buffers[3,33,38]. Two structural properties are of importance for the occurrence of complex formation with boric acid and therefore for the migration in the electric field[3]. These are:

(1) Hydroxyl groups in the *cis*-positions ("Böeseken complexes"[15]); this is the commonest case and is to be expected from previous observations.

(2) "Tridentate complexes" (see the adjacent formula).

The formation of such compounds results in six-membered rings with hydroxyl groups in the 1, 3 and 5-positions in axial arrangement. The mobility of *cis*-phloroglucitol and *scyllo*-quercitol, and the very small loss in mobility of 2- and 4-O-methylated *myo*-inositol is thus explained (see Table 11).



Tridentate complex of *cis*-phloroglucitol.

Purines, nucleosides and nucleotides

This class of compounds has often been investigated by paper electrophoretic methods although generally only relatively small potential gradients have been employed[19,20,60,77,112,128,132]; further information is given in various monographs[14,72,78,122,143,144].

TURBA *et al.*[125] first separated the three adenosine phosphoric acids and several nucleosides in a pyridine–acetic acid buffer of pH 6.45 by means of high potential gradients. CRESTFIELD AND ALLEN[28] examined various nucleotides in phosphate and borate buffers; they attempted to separate the 3′ and 5′ phosphorylated compounds by the diverse possibilities of complex formation with boric acid.

MICHL AND HABERLER[88] determined the content of purines and methylated xanthines in various drugs by means of HVE, spectroscopic methods and colour reactions. Adenine and guanine could easily be separated at pH 3.6 and stained with a bromophenol blue reagent[88]. The xanthine derivatives were separated at pH 10.6 (see Table 12).

Further quantitative information on the migration rates of various compounds which belong to this class and which can be rapidly separated by HVE, is to be found in the literature and in the references given above. Only in the case of compounds which are readily adsorbed by filter paper, *e.g.* some guanidine derivatives, the migration rate is sometimes reduced. The values of WADE AND MORGAN[128] which are given in Table 13 can be reproduced under the conditions of HVE.

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TABLE 11[3]

RATIO OF MOBILITIES OF CYCLITOLS/GLUCOSE

Apparatus according to FOSTER[32]; about 20 V/cm; 0.012 *M* borax solution

Cyclitol	*Position of OH-groups*	*Ratio*
Inositol:		
scyllo-Inositol	(1:3:5/2:4:6)	0.02
(+) Inositol	(1:2:4/3:5:6)	0.28
neo-Inositol	(1:2:3/4:5:6)	0.30
myo-Inositol	(1:2:3:5/4:6)	0.30
muco-Inositol	(1:2:4:5/3:6)	0.87
allo-Inositol	(1:2:3:4/5:6)	0.54
epi-Inositol	(1:2:3:4:5/6)	1.50
cis-Inositol	(all *cis*)	1.60
Quercitols:		
scyllo-Quercitol	(1:3:5/4:6)	0.05
proto-Quercitol	(1:3:4/2:5)	0.05
vibo-Quercitol	(1:2:4/3:5)	0.07
epi-Quercitol	(1:2:3:5/4)	1.17
cis-Quercitol	(all *cis*)	1.60
Inositol methylethers:		
1-Methyl-*myo*-inositol (Bornesitol)		0.02
2-Methyl-*myo*-inositol		0.29
4-Methyl-*myo*-inositol (Ononitol)		0.45
5-Methyl-*myo*-inositol (Sequoyitol)		0.05
2-Methyl(—)-inositol (Quebrachitol)		0.14
3-Methyl(+)-inositol (Pinitol)		0.23
1:3-Dimethyl-*myo*-inositol (Dambonitol)		0.00
Cyclohexanetriols:		
1:3/2 Cyclohexanetriol		0.00
1:2/3 Cyclohexanetriol		0.08
cis-1:2:3-Cyclohexanetriol		0.05
cis-1:3:5-Cyclohexanetriol (Phloroglucitol)		0.11
Cyclohexanediols:		
trans-1:2-Cyclohexanediol		0.00
cis-1:2-Cyclohexanediol		0.01
trans-1:3-Cyclohexanediol		0.00
cis-1:3-Cyclohexanediol		0.00

TABLE 12[88]

RATIO OF DISTANCES OF MIGRATION OF COMPOUND/XANTHINE

In 1 *N* ammonia:1 *N* acetic acid *ca.* 20:1; pH 10.6; 40 V/cm

Compound	*Ratio*
Caffeine	0.00
Theobromine	0.44
Hypoxanthine	0.83
Xanthine	1.00
Theophylline	1.14

TABLE 13[128]

RATIO OF DISTANCES OF MIGRATION OF NUCLEOTIDES/ORTHOPHOSPHATE

1 l buffer contains 92 ml butyric acid (*ca.* 1 *N*) and 1 g NaOH (0.025 *N*); pH *ca.* 6.4; 10 V/cm

Nucleotide	*Ratio*
Adenosine-2′-phosphate	0.23
Adenosine-3′-phosphate	0.23
Adenosine-5′-phosphate	0.21
Adenosine-5′-diphosphate	0.58
Adenosine-5′-triphosphate	0.86
Cytidine-2′-phosphate	0.13
Cytidine-3′-phosphate	0.13
Dihydrodiphosphopyridine nucleotide	0.48
Diphosphopyridine nucleotide	0.13
Flavine-adenine dinucleotide	0.36
Flavine mononucleotide	0.36
Guanosine-2′-phosphate	0.42
Guanosine-3′-phosphate	0.42
Orthophosphate ion	1.00
Uridine-2′-phosphate	0.55
Uridine-3′-phosphate	0.55
Uridine-5′-phosphate	0.55

TABLE 14[10]

APPARENT MOBILITIES OF ORGANIC ACIDS

In $cm^2\ V^{-1}\ sec^{-1}$ (defined by WEBER[130]); dilute formic acid of pH 2.0; apparatus according to BERBALK[10]

Acid	$K \cdot 10^4$	*Apparent mobility*
Picric acid	3810	6.1
Oxalic acid	650	9.0
Dichloroacetic acid	330	6.5
Maleic acid	120	6.7
Pyruvic acid	30	3.7
Monochloroacetic acid	15	2.9
Aconitic acid	15	2.4
Fumaric acid	10	2.2
Tartaric acid	9.8	2.3
Citric acid	8.3	1.9
Sulphanilic acid	6.2	1.6
Malic acid	3.8	1.4
Glyceric acid	2.3	1.2
Glycolic acid	1.5	0.7
Itaconic acid	1.5	0.7
Lactic acid	1.4	0.7
Succinic acid	0.64	0.2
Glutaric acid	0.47	0.1
Adipic acid	0.33	0.1
Valeric acid	0.16	0.05

Organic acids

Organic acids which are soluble in water can be satisfactorily separated electrophoretically provided that their dissociation constants are sufficiently different[79,138]. Mixtures of difficultly or non-volatile hydroxy- and dicarboxylic acids are particularly simple to investigate. Formic acid (*ca.* 0.55 *N*; pH 2.0)[10] is used as solvent for stronger acids, and pyridine-acetic acid (pH 4.1)[87] for weaker acids. The acids can readily be detected after evaporation of the buffer by means of indicators or by other usual paper chromatographic methods. Stable colorations (see Fig. 7) can be achieved by a kind of reversed sugar test[87]. Several examples are given in Table 14 and Fig. 7.

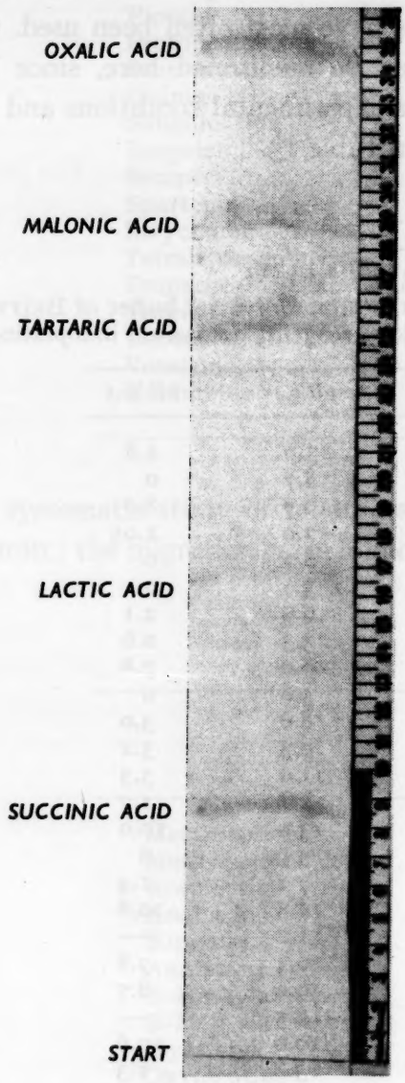

Fig. 7. Apparatus as in Fig. 3; 40.3 V/cm, 2.3 mA/cm of the width of the strip; 90 min; 10°. Solvent: 1.5 ml pyridine, 5.5 ml glacial acetic acid, 15 ml acetone diluted to 100 ml with water (pH 4.1). Colour reaction: 10 mg glucose, 5 mg arabinose dissolved in 3 ml ethanol and 0.5 ml aniline and 3 ml butanol added; after spraying, the strip is heated to 140°[87].

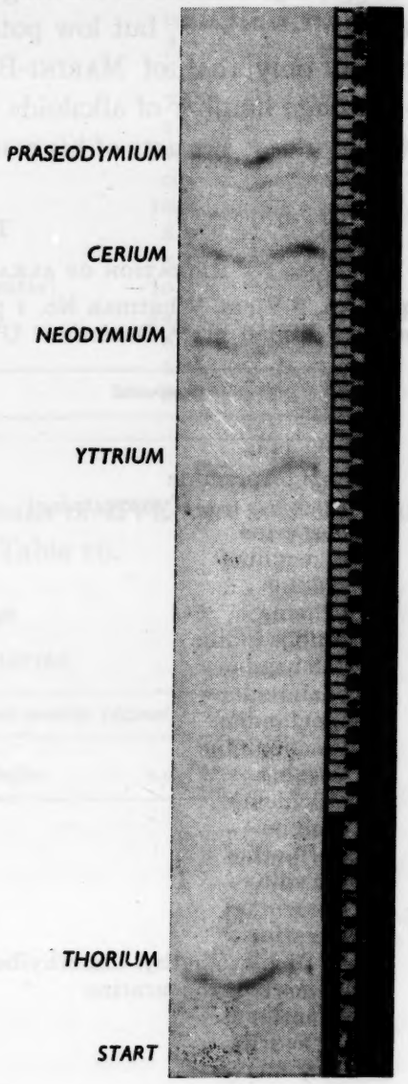

Fig. 8. Apparatus as in Fig. 3; 46 V/cm, 1.1 mA/cm of the width of the strip; 90 min; 10°. Solvent: 15 ml acetone diluted to 100 ml with 0.1 *N* lactic acid. Colour reaction: 0.2% catechol violet in 2 *N* ammonia; after spraying, the excess is removed by washing with distilled water[87]

(See p. 34).

References p. 35.

Further investigations have been carried out by GROSS[47] particularly on the acids which often occur in biological materials. The separation of higher fatty acids in 2 *N* ammonia, as described by BARNETT AND SMITH[7], is unsuccessful when HVE is used because of excessively strong adsorption phenomena. The separation of humic acid mixtures is usually very difficult but was achieved by SCHEFFER *et al.* by HVE and cooling with chlorobenzene[106].

Alkaloids

If the alkaloids are sufficiently soluble in water, they too can readily be examined electrophoretically. Numerous investigations have been carried out (for details see the monographs[14,72,78,143,144]), but low potential gradients have most often been used. Of these studies, only that of MARINI-BETTOLO[75,76] need be mentioned here, since he examined a large number of alkaloids under the same experimental conditions and at different pH-values; extracts of his results are given in Table 15.

TABLE 15[75]

MIGRATION OF ALKALOIDS TO THE CATHODE IN cm

LKB-apparatus, 8 V/cm, Whatman No. 1 paper, duration 180 min. Universal buffer of BRITTON AND ROBINSON diluted 1:4[99]. Detection: UV-light, Dragendorff reagent, potassium iodoplatinate

Compound	*pH 6.4*	*pH 10.5*
Arecoline	13.6	2.8
Aspidospermine	8.7	0
Atropine (DL-Hyoscyamine)	10.7	8.0
Berberine	2.0	1.05
Bicuculline	6.2	0
Boldine	3.0	0
Brucine	6.9	2.1
Bulbocapnine	3.3	0.6
Calebassine	8.0	7.8
Chelidonine	5.8	0
Cinchonine	8.0	3.0
Cinchonidine	9.3	3.2
Cocaine	11.0	5.3
Colchicine	2.1	1.7
Coniine	13.0	10.0
Corlumine	5.9	0
Corydine	7.1	1.4
Cotarnine	12.5	10.8
Curarine	11.0	—
N-Dichloromethyl-dimethylberberine	10.4	7.8
Dimethyltubocurarine	9.9	8.7
Emetine	8.5	—
Ephedrine	10.0	10.6
Ergometrine	3.8	1.3
Eserine	10.5	—
Fluorocurine	8.0	8.0
Galegine	11.8	10.8
Glaucine	3.8	0.3
Harmine	2.6	0
Heroin	9.5	2.85
Homatropine (Mandelic acid tropylester)	10.4	10.0
L-Hyoscyamine	10.3	6.9
Hydrastine	6.1	0

TABLE 15 (Continued)

Compound	*pH 6.4*	*pH 10.5*
Hydrastinine	13.2	11.3
Jervine	5.3	0
Lupanine	10.6	8.7
Lupinine	12.1	10.5
Mescaline	10.2	8.2
Morphine	10.0	2.85
Narceine	2.6	1.65
Narcotine	5.4	0
Nicotine	12.3	4.5
Papaverine	3.0	0
Pilocarpine	10.6	1.95
Piperine	0.4	0
Protopine	7.7	3.9
Protoveratrine	6.5	—
Quinine	7.6	2.5
Sanguinarine	1.1	0.0
Scopolamine	10.4	3.3
Sempervirine	0.5	0.0
Sparteine	10.8	9.9
Strychnine	8.5	0.0
Tetrahydropalmatine	4.1	0.0
Tropacocaine(Benzoic acid pseudotropylester)	10.2	8.8
Tropine	14.5	0.0
Tubocurarine	8.4	4.7
Veratrine	6.2	1.4
Yohimbine	6.8	1.0

A systematic study of tobacco alkaloids by means of HVE was made by KUHN AND MICHL; the migration rates found are shown in Table 16.

TABLE 16[90]

APPARENT MOBILITIES

Alkaloid	*Apparent mobility (defined[130]) at 10°*	
	pH 5.0 K-biphthalate buffer	*pH 9.2 0.025 M borax solution*
Metanicotine	12.7	7.7
Methyl-metanicotine	12.5	7.0
Nornicotine	12.2	7.0
Anabasine	10.7	6.1
Nicotine	12.1	2.5
Anatabine	10.5	2.3
N-Methylanabasine	10.1	0.8
N-Methylanatabine	10.0	0.8
Myosmine	8.0	0.8
Oxynicotine	3.9	0.7
Nicotone	3.4	0.7
Nicotyrine	3.2	0
Nornicotyrine	1.5	—0.7

It is possible to recognize from these values a definite relationship between the electric migration rate and the chemical constitution. Thus compounds containing a

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secondary nitrogen atom (nor-compounds) migrate more quickly than the corresponding compound with a tri-substituted nitrogen atom; nornicotine moves more rapidly than nicotine, anabasine than N-methylanabasine, etc. Moreover, compounds containing an aliphatic side-chain, such as metanicotine and N-methylmetanicotine, migrate more quickly than the corresponding cyclic derivatives. Tobacco alkaloids with double bonds in the second ring—the first is certainly a pyridine ring in all these cases—show a smaller mobility than those with saturated systems; thus anatabine (piperidene ring) is slower than anabasine (piperidine ring).

Nicotyrine and nornicotyrine behave irregularly owing to adsorption phenomena. If these compounds are subjected to paper chromatography with the same buffers as used in HVE, they give R_F-values of 0.66 and 0.37 respectively as against about 0.9 for the other substances; thus it seems comprehensible that their migration rates will be correspondingly smaller. This phenomenon is also encountered quite frequently with other alkaloids. In the examination of pharmaceuticals[104], cocaine migrates between codeine and morphine whereas more rapid migration would be expected on the basis of the dissociation constants.

Inorganic ions

Examples of the investigation of inorganic materials by means of HVE are only infrequent. The writer[79] has separated copper(II) from cadmium(II); unexpectedly the former migrated more quickly than the latter. WERNER AND WESTPHAL[134] obtained different migration rates for iron(II), cobalt(II), cadmium(II), lead(II), copper(II) and iron(III) in a formic acid–acetic acid mixture. SCHIER[107] was able to resolve a mixture of lithium, sodium and potassium ions and to determine each ion quantitatively. WIELAND AND PFLEIDERER[141] investigated a number of derivatives of phosphoric acid.

Most of the numerous investigations which have been carried out by low voltage electrophoresis (for details, see the monographs, especially that of LEDERER[72]) could also be done by HVE, which would often increase considerably the amount of separation. For example, a mixture of several rare earths can easily be separated by methods described in the literature[70,71,105] (Fig. 8, p. 31).

The separation of isotopes by these methods has also been examined[58]. BONNIN *et al.*[16] accomplished the partial separation of ^{22}Na and ^{24}Na in this way.

5. FURTHER POSSIBILITIES

It has been frequently emphasized already that the possibilities of application of HVE are by no means confined to the classes of compounds mentioned above. The introduction of HVE should improve and accelerate the separation of most of the substances of low molecular weight which previously have been examined by paper electrophoresis.

It is very convenient that the rates of migration can be estimated in advance if the dissociation constants of the substances are known. Conversely, it is possible to estimate approximately unknown dissociation constants by comparison with known compounds, and only a very small amount of material is required. Moreover, the dissociation constant

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of an initially unidentified substance can be estimated and then appropriate compounds can be sought from tables, for the class of the compound is already known from the colour reactions applied. For example, in this Institute, organic acids produced by the decomposition of natural materials with potassium permanganate have repeatedly been detected by this method[87].

Conclusions can readily be drawn on the presence or absence of carboxyl- or amino groups in unknown organic materials from the direction of migration. Adjacent hydroxyl groups may be detected by their reaction with borate[59], and in many cases, carbonyl groups can be recognized by their migration in buffers containing bisulphite.

HVE has already established its value in work on organic synthesis. For example in the synthesis of tosylarginine methylester from arginine, four reaction products, *viz.*, arginine, arginine methylester, tosylarginine and tosylarginine methylester, are obtained; KICKHÖFEN[64] separated these four compounds in less than 50 minutes at pH 7.3 and 60 V/cm. In another case, he methylated histidine with methyl iodide in liquid ammonia; the three reaction products, 1-methylhistidine, 3-methylhistidine and the starting material, could be separated in 2½ hours[64]. When the Kolbe synthesis with β-naphthol is used, a mixture of, for example, β-naphthol-1-carboxylic acid and β-naphthol-3-carboxylic acid is obtained; WIELAND AND PFLEIDERER[141] showed that these could be separated in less than 3 hours. The same authors used HVE to analyse the products of the nitration of phenols and several other mixtures originating from the syntheses of organic compounds[141].

Previously in such investigations, buffer solutions which are predominantly aqueous have been used. However, many groups of compounds which have hitherto been neglected could be examined if solvents containing little or no water were applied. Further work is required in this direction.

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ADDITIONAL LITERATURE

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A METHOD FOR THE PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF PHENOL DERIVATIVES, MOULD METABOLITES AND RELATED COMPOUNDS OF BIOCHEMICAL INTEREST, USING A "REFERENCE SYSTEM"

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I. INTRODUCTION

When dealing with the problem of correlating the production of different metabolic compounds, found in various strains of *Penicillia*, with their general biosynthetic pathways, great difficulties are encountered in the identification of these compounds, especially when they only occur in minor amounts. The pioneering work on mould-metabolic products as carried out by RAISTRICK and his colleagues (for reviews see Refs.[1,2,3]) led to the isolation and identification of a great number of compounds, such as phenol derivatives, tetronic acids, tropolones, etc., from large scale cultures of appropriate moulds by chemical methods.

In order to compare the production capacity of a number of different strains under various conditions, such as addition of antimetabolites or isotopes, it was found favourable, at least in preliminary experiments, to cultivate the moulds on a relatively small scale (100–500 ml of media). On the basis of preliminary paper-chromatographic studies of medium extracts from some *Penicillia* strains (*e.g. P. urticae* Bainier, *P. brevi-compactum*, *P. griseo-fulvum* etc.), EHRENSVÄRD[4,5] showed that 2–3 times as many compounds of "aromatic" character could be detected, as had been reported previously.

It was felt that when substances were found in the culture media (or in mycelia) even in very small amounts compared with the "main" products, these could, after proper characterization or identification, provide additional information regarding the possible precursors or reaction sequences in the biosynthesis of accumulated compounds.

As a basis for the investigations, a suitable "reference system" was worked out for comparative purposes. Since, as previously stated, mould-metabolic products are mainly related to the phenol or phenol-carboxylic acid type of compounds, the reference system had to cover a large number of the most simple phenol derivatives so that possible degradation and decarboxylation products could be detected. Furthermore, by courtesy of Prof. RAISTRICK, it was possible to include a large number of well-known mould metabolites. It was also found useful to collect a large group of non-aromatic compounds, mainly related to the TCA-cycle, and some of the more

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common natural products of vegetable origin, using the same procedure. The choice of substances was, of course, somewhat arbitrary, within the limits of their availability. In many cases the heterogeneity of the compounds proved to be of help in determining the group character of certain types of compounds.

The common paper-chromatographic procedure had to be adapted both to the investigation of the reference substances and to that of the extracts from culture media of moulds. This was done according to the following principles:

The chromatograms were run in six different solvent systems. Each of these was treated as a standard with ten spraying reagents. The limited choice of solvents and reagents was conditioned by the need for obtaining, within a reasonable time, adequate information on the chemical nature of the compounds involved.

To avoid the tedious and time-consuming task of spraying only one chromatogram at a time, a set-up was constructed that allowed six chromatograms at a time to be sprayed with ten standard reagents within a relatively short time (Fig. 1). The principle of this system is to apply the extract along a line on each of the six chromatographic papers and then develop them in six solvents. After drying, they are sprayed vertically with the ten reagents in adjacent strips (about 10 mm wide). When the level to which the substance has travelled is crossed by the vertical reagent strip, a roughly circular spot appears. From this the R_F value can be read off directly by means

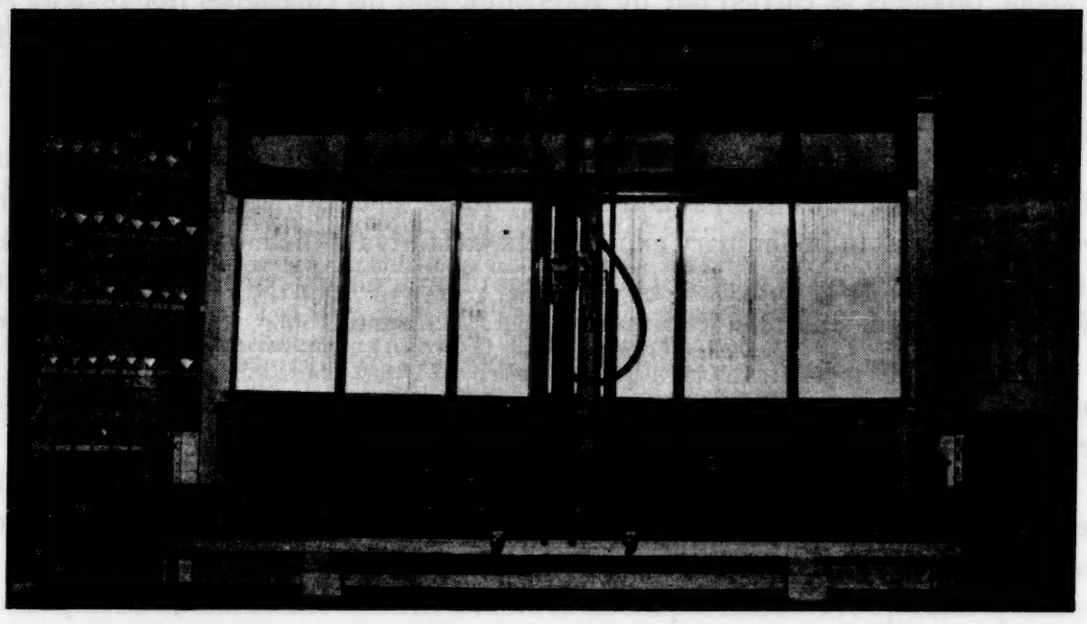

Fig. 1. Over-all picture of the spraying arrangement, placed on an 80 cm high table. Six papers from chromatographic solvent systems can be inserted simultaneously against an illuminated glass screen. The first section from the right shows that it is possible to spray at least 22 strips of reagents, vertically, on each paper of size 24 × 55 cm. The frame shown in the middle of the figure can be moved both horizontally and vertically. It is equipped with two retouching air brushes and is counterbalanced so as to allow an easy up and down movement when spraying. Between the air brushes and the paper there are two slits, 8 × 500 mm, which can only be moved horizontally together with the frame. At both sides of the plate where the slits are fixed, a graduated elastic band is attached, which is protected against sprinkling by an acrylate plate. The R_F values are read off directly and recorded after the band has been properly adjusted, by means of a screw, to the solvent front on the chromatogram.

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of a graduated elastic band. At the same time the shades of the colours produced by the action of the different reagents are recorded in numbers. For these colours, an arbitrary standard has been chosen according to the "Derwent" colour pencil shades for rapid recording (see the colour index given for the tables). When the colours are fully developed, the variation of the R_F values of a single component, which are represented horizontally by similar colour sequences in the six solvents, can easily be studied against an illuminated screen. An over-all idea of the results can thus be obtained during spraying. This was found to be advantageous in many cases when checking only the "typical" production picture of a single strain. For a complete record of the numerical data, consisting of R_F values and of a numbered code of colour reactions, the reader is referred to the experimental part of this work.

This system of characterizing unknown substances before attempting to isolate them in a crystalline form, has hitherto resulted in the identification of several new metabolites. For instance, GATENBECK[6] studied the biosynthetic background of anthraquinones and reported the presence of 3-hydroxyphthalic acid in *Penicillium islandicum* Sopp. In the culture media of *P. griseo-fulvum*[7] I detected, among other substances, orcinol and orsellinic acid, which have not been reported previously either in the case of *Penicillia* species or of any other moulds. Penicillic acid, which had earlier been found in several other species of *Penicillia*, was also shown to occur in this species. After isolation and chemical analysis of the three above-mentioned compounds, their identity was confirmed. LYBING AND HAGSTRÖM[8] described an interesting application of this method in an investigation of secretion products in egg water from sea urchin eggs.

2. GENERAL PROCEDURE

Extracts or mixtures of several compounds, which were subjected to paper-chromatographic investigation were run on Whatman No. 1 filter papers, 24 × 55 cm, in six different solvent systems designated as F, E, A, B, C and D. By drawing a funnel-shaped pen (Fig. 5) along a ruler, various substances in ethanolic solution were applied uniformly on the papers along a line 20 cm in length and 10 cm from the bottom of the paper. The line was dried and the procedure was repeated until the desired concentration of substances was obtained. On both sides of the line on each paper, 2 spots of a "standard" substance were applied in order to maintain a continuous control of the quality of the paper and the constancy of the solvent systems. Usually an extract of about 1 mg dry weight, containing up to 6 components was applied on one paper. However, in most cases it was advisable to test several concentrations in order to get the best separation. In the case of one substance only, the procedure was simplified and a line chromatogram was run in one solvent only. In the remaining five solvents, the substance was applied and run as a spot and developed using the best detection reagent, which was found by spraying a line chromatogram with 10 standard reagents. When there was less than 0.1 mg substance, the procedure could be modified so as to comprise spot-test reactions on filter paper and estimation of the R_F values for 6 spots, one for each solvent system.

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The chromatographic papers were hung up for 1 h in the chromatographic jars for equilibration before the solvents were applied. After the chromatograms had been run for approx. 4 h in solvents F, E, A, and for 5 h in solvents B, C and D, the solvent front had reached a distance of about 40 cm from the starting line; the solvent front was then marked by a soft pencil. The papers were air-dried overnight at room temperature. This gave the best results when the indicator reagent was used for detecting acidic components. Spraying could even be performed after 2-hours' drying, but in order to get a positive response with the indicator reagent, this should be applied 3 times, followed by ammonia and $CuSO_4$, similarly to the procedure recommended for solvent system E. In the case of the other reagents, the drying time had no noticeable effect.

The six chromatograms were placed in the order F, E, A, B, C, and D against an illuminated screen (as shown in Fig. 1), where they remained during spraying and evaluation. After examining the chromatograms in U.V.-light, they were sprayed with 10 different reagents, and the colours were recorded. For detailed descriptions see the sections describing spraying technique, and colour estimation and recording respectively.

3. THE SPRAYING APPARATUS

The spraying apparatus was built from L-profile aluminum and ½ inch aluminum rods. The dimensions of the frame were: length 200 cm, height 120 cm and depth 35 cm. An earlier model constructed mainly from laboratory clamps and rods also proved satisfactory. In the middle of the frame, 6 separate sections were set up, corresponding to the size of the papers used, each provided with a glass plate. The sections were arranged so that they could be taken out for cleaning, by pushing them sideways. Each section consisted of a rectangular unit, made from L-profile stainless steel, on the inside of which the above-mentioned glass plate was fitted. Between the inside border of the L-profile unit and the glass a gap of about 1 mm is obtained when the glass plate is pushed backwards. The chromatogram was fastened by pushing the paper between the glass plate and the overlapping (10 mm wide) L-profile border surrounding the unit. No special clamps or tape had to be used to hold the chromatogram upright. This applies also to papers of other sizes, but of the same length.

Behind these sections two daylight lamps, each 1.5 m long, were built in, so as to avoid corrosion of the lamps. In order to secure maximum light intensity and a uniform light source (whereby colour photographs could also be taken), the lamp section was coated on the inside with aluminum foil. The upper part of the spraying apparatus was insulated against fluids and packed with absorbent filter paper in order to collect the rinsing-spray fluids. It is also recommended to connect this with a ventilation device.

At the front of the spraying apparatus there is a mobile rack carrying two air brushes, as shown in Fig. 2. For spraying, this is moved vertically; it can also be shifted horizontally from one paper to another, at a constant distance from the paper. The tips of the air brushes are fixed 9 cm from the surface of the paper. A plate coated with filter paper and provided with two adjustable slits, 8 × 500 mm, was placed

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between the chromatogram and the two air brushes, 1 cm from the paper surface. This could be moved since it was connected with the rack.

On both sides of the plate with the two slits, a graduated elastic band for R_F measurements was fixed. This could be adjusted with a screw to follow the variations of the solvent front on the chromatogram.

The R_F values for the centre of gravity of the single spots were read off by moving

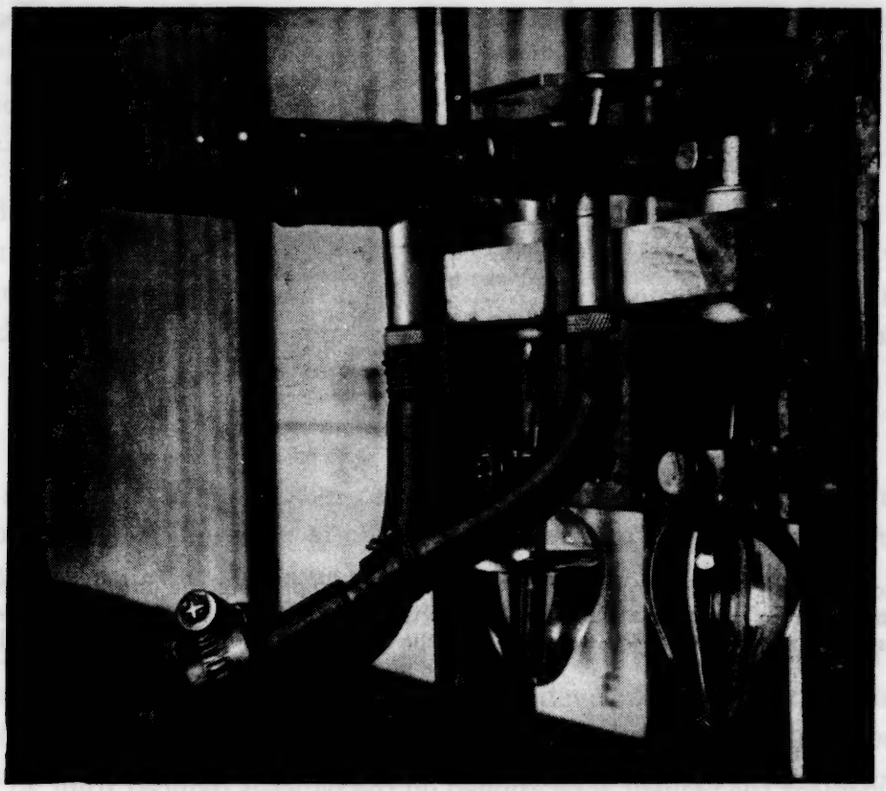

Fig. 2. Detail of that part of the movable frame to which the two air brushes and their reagent flasks are attached on an acrylate plate.

the rack horizontally adjacent to the spots. In some cases the R_F ordinates were drawn on the chromatograms in pencil.

The spraying screen was provided with a movable ultraviolet lamp. When turning the lights off (in a dark room), the fluorescent spots were outlined in pencil and the colour was recorded.

Fig. 1 shows a rack holding the reagents (left) and a colour index (right).

4. SPRAYING TECHNIQUE

For spraying, two "Grafo" Retouching Air Brushes, Type IIB were used (Fig. 3). These were operated with compressed air, but compressed nitrogen or carbon dioxide may also be used. The minimum working pressure being 2 atm, the air brushes were operated at 2.4 atm.

The sprayers were fitted on a mobile rack, which allowed horizontal and vertical

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movement at a constant distance from the chromatographic papers. The reagents were sprayed vertically one after another from the vessels containing 15 ml of the reagent solution. For continuous spraying during 2½ min approx. 15 ml of solution was required (tested with aqueous solutions). This is sufficient to allow the spraying of 60 strips, each 50 cm long, *i.e.* corresponding in all to 30 m chromatogram. The amount of spraying solution can be regulated to a certain extent, as can be seen in Fig. 3. Under normal conditions the spraying cone is adjusted so that about a 20 mm wide line of reagent is applied on the paper. Between the air brushes and the paper there are two

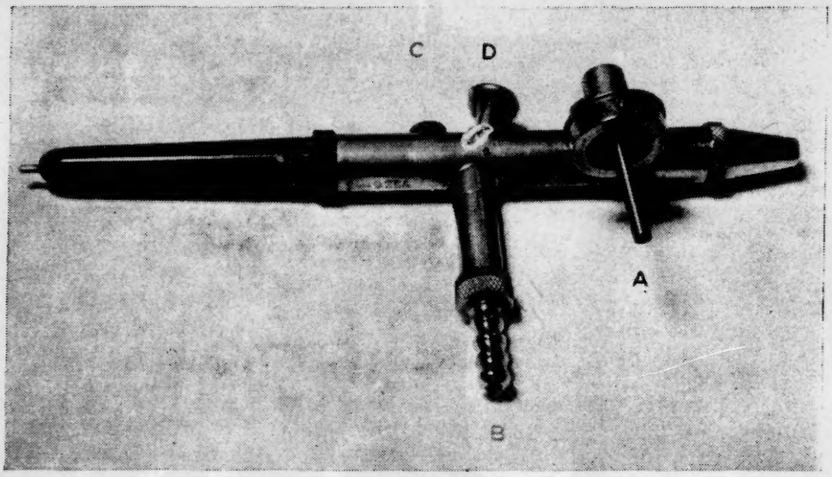

Fig. 3. Retouching air brush, type II B "Grafo", used for spraying the chromatograms. A: Inlet for reagents, which in this special case has been extended to reach the bottom of the reagent flask. B: Inlet for compressed air. C: Screw to regulate the quantity of solution sprayed. D: Push button to start the sprayer.

slits, 8 × 500 mm, which can only be moved horizontally together with the frame. By this arrangement the reagent zone applied on the paper is reduced from 20 to 10 mm.

When one line has been sprayed on the first paper at a certain position, the sprayer is moved to the next paper and, at the same position, another line is sprayed. After the six chromatograms have been treated similarly the reagent solution is changed. The sprayer is washed for 10 sec with distilled water (or alcohol, depending on the reagent used or the one to be used).

Since the air brushes are precision instruments, the following precautions should be carefully observed:

The reagent solutions must be protected from dust and other visible impurities. Before pouring them into spraying-flasks, they should be filtered. When not in use they should be stored in tightly closed flasks as shown in Fig. 1. If the solutions become turbid, they must be refiltered.

The air brush must be carefully cleaned after use by blowing water of *ca.* 50° through it, especially when ammonia or phosphomolybdic acid have been used. Distilled water should be used for cleaning the brush between sprayings. Ethanol should be used before and after the reagent DB (= 2,6-dibromoquinone-4-chloroimide), otherwise the reagent will precipitate and block the channels in the air brush.

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In order to facilitate successive sprayings with two different reagents within the same strip, the following spraying scheme has proved useful:

Reagents	
Air brush I	*Air brush II*
Bromophenol blue	$CuSO_4$, for paper from solvent E only
—	Permanganate
Ferric chloride	—
Dinitrophenylhydrazine	—
Phosphomolybdic acid	Ammonia
D1, D2, D3 and D4 diazonium reagents	Ammonia
2,6-Dibromoquinone-4-chloroimide	Ammonia

Reagent flasks (15 ml) are hung up by two metal clamps on the rack to the left of the spraying arrangement (Fig. 1). The upper clamp supports the cap-type polyethylene stoppers and the lower holds the reagent flasks, which can thus be easily removed and transferred from the rack to the spraying apparatus and vice versa with one hand only.

5. SPRAYING REAGENTS

Diazotized sulfanilic acid = D1. 50 mg stable diazonium salt was dissolved in a mixture of 5 ml dioxane and 10 ml water at 0° and filtered. When not in use, the solution was stored in a refrigerator at the same temperature.

With most phenolic compounds and aliphatic keto acids, this reagent gave a yellow to orange colour—in very few cases a pink colour was produced—on exposing the sprayed strip to ammonia. Sometimes a spot developed even without ammonia.

Diazotized 4-benzoylamino-2,5-dimethoxyaniline = D2. 100 mg stabilized Zn salt was dissolved in a mixture of 5 ml dioxane and 10 ml water at 0°, filtered and stored in a refrigerator.

With the investigated phenolic compounds this reagent gave purple to red-violet colours—in a few cases yellow and brown colours were obtained—when the sprayed strip was exposed to ammonia.

Diazotized o-dianisidine = D3. 100 mg stabilized Zn salt was dissolved in a mixture of 5 ml dioxane and 10 ml water at 0°, filtered and stored in a refrigerator.

With the investigated compounds this reagent gave red to blue-violet colours —in a few cases brown or blue colours were obtained—when the sprayed strip was exposed to ammonia.

p-*Nitrobenzenediazonium fluoroborate = D4.* 60 mg dry diazonium salt was dissolved in a mixture of 5 ml dioxane and 10 ml water at 0°, filtered and stored in a refrigerator.

With the investigated compounds this reagent produced an orange-yellow colour—in some cases brown, violet and red colours were produced—on exposing the sprayed strip to ammonia. Very small amounts of ammonia are required to produce this reaction which is, in most cases, immediate.

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2,6-Dibromoquinone-4-chloroimide = *DB*. 50 mg 2,6-dibromoquinone-4-chloroimide was dissolved in a mixture of 12 ml CaH_2-dried dioxane and 3 ml dry acetone. The solution was filtered and stored in a refrigerator.

With phenolic compounds this reagent gave blue, green and gray colours—in some cases yellow and pink colours were obtained—on spraying the reagent strip with dilute ammonia.

Solutions of all the diazonium reagents and the DB-reagent were stored in pear-shaped flasks of 15 ml on a separate rack, which could easily be moved over to a refrigerator after spraying. The reagent solutions could be used up to 14 days after preparation. However, every day the background colour (blank test) increased in intensity. Dioxane was used in order to dissolve the diazonium salts more easily; cold 50% aqueous ethanol could also be used, but the solutions were then found to lose their activity after a week. Dioxane was purified from peroxides by treatment with CaH_2 for a few days and subsequent filtration. For suppressing the original colour in the D2 and D3 solutions, treatment with active carbon was successful, but afterwards the solutions remained stable only for a couple of days, owing to the change of pH in the solution. Addition of small amounts of acetic acid had no effect on the storing stability of the solutions.

Ferric chloride = *Fe*. 2% $FeCl_3 \cdot 6H_2O$ in water was used. If storing for longer times is desired, then the stock solution must be at least 6%. The use of saturated solutions of anhydrous ferric chloride in anhydrous dioxane or chloroform may sometimes be advantageous.

With the investigated compounds this reagent gave red-violet, blue or green colours, sometimes also brown, gray and yellow.

2,4-Dinitrophenylhydrazine = *DN*. 500 mg 2,4-dinitrophenylhydrazine was dissolved in 1000 ml hot 1 *N* HCl. The solution was filtered after a few days.

With aldehydic and keto compounds, this reagent gave yellow to orange colours; in rare cases other substances gave a positive reaction.

Potassium permanganate = *Mn*. This reagent consists of 1% aqueous solution of potassium permanganate. In some cases the use of slightly alkaline or acid permanganate was preferred.

With the compounds investigated, this reagent gave yellow-brownish spots on a red-violet background. Sometimes the spots were colourless owing to the strong reduction of the reagent to Mn^{+2} ion.

Phosphomolybdic acid = *Mo*. 2% aqueous solution of $H_3PMo_{12}O_{40} \cdot 29H_2O$ was used. In this case also the strip was sprayed with ammonia in order to develop the colours. Sometimes the colour development was immediate, even if ammonia was not used, and sometimes the colours changed when the reagent strip was exposed to ammonia. With the compounds investigated this reagent gave blue and gray colours; in rare cases yellow or green spots were obtained.

Bromophenol blue = *Ind*. 300 mg bromophenol blue was dissolved in 500 ml ethanol and 0.25 ml 30% sodium hydroxide was added in order to change the colour of the solution from red to blue.

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With acids, this reagent gave yellow spots on a blue background. Chromatograms from the solvent system E did not give spots with the indicator reagent owing to the alkali remaining in the paper. In this special case, the bromophenol blue strip was once again sprayed with 2% aqueous solution of $CuSO_4 \cdot 5H_2O$. The background then turned to light blue or gray and the spots obtained, indicating the position of acids, were blue, green or yellow.

Dilute ammonia. Dilute ammonia was used when spraying the diazonium reagents D1, D2, D3, D4 and DB and Mo. In the cases previously mentioned, the dilute ammonia solution was made up as follows: 1 part concentrated aqueous ammonia (25%) and 1 part ethanol.

Copper sulphate. This reagent was used for the detection of acids when bromophenol blue failed to react. The paper was first sprayed with a 2% solution of $CuSO_4 \cdot 5H_2O$ in water and then with dilute ammonia. Practically all aromatic and aliphatic acids gave blue, green or brown spots on an almost colourless background.

Ninhydrin reagent = NH. Amino acids and biogenic amines were detected by spraying with 2% ninhydrin in butanol saturated with water and subsequent heating with an electric dryer.

Additional spraying reagents

For aliphatic acids. Aliphatic acids can also be detected by spraying with 1:4 diluted D3 reagent (*o*-dianisidine-diazotate) and subsequent spraying with 0.1% β-naphthol in 0.02 *N* sodium hydroxide. In this case the spots were almost colourless and the background turned dark violet[9].

The following stabilized diazonium compounds were also tested, both with regard to their usefulness for detecting phenolic compounds and the possibilities they offer for distinguishing between several compounds on paper chromatograms. About 0.7% solutions of diazonium compounds in 1 part dioxane and 2 parts water were used. These were: diazotized 4-amino-2-chlorodiethylaniline $ZnCl_2$ salt, *o*-aminoazotoluene-diazotate, anthraquinone-1-diazonium chloride, diphenylamine-4-diazonium sulphate, 1-diazo-2-naphthol-4-sulphonic acid, NNCD-reagent = 2-chloro-4-nitrobenzenediazonium naphthalene-2-sulphonate (previously described as reagent in paper chromatography[10]) and tetrazotized *o*-dianisidine. The latter shows colour reactions which are similar to those of *o*-dianisidine-diazotate. However, the shades of the colours were deeper.

"Genochrome" reagent. 2% aqueous solution of *p*-aminodiethylaniline sulphur dioxide was prepared. This reagent was stable at room temperature for two weeks. When spraying with this reagent aromatic aldehydes immediately gave orange to red spots on a colourless background. The latter turned brown-gray after several hours. Aromatic ketones and phenols do not react under these conditions. Other applications of the "Genochrome" reagent have been described in ref.[11].

Phenolic compounds gave yellow, violet, blue and brown colours when the reagent strip was sprayed the second time with 0.1% aqueous solution of sodium periodate.

Bromophenol blue and hydroxylamine hydrochloride reagent. 150 mg hydroxyl-

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amine·HCl was dissolved in 14 ml bromophenol blue solution (Standard Indicator solution) and 1 ml water. The pH of the solution was adjusted to 7.2 with a few drops of 2 *N* NaOH. Aromatic aldehydes reacted rapidly and gave yellow spots on a blue background. Ketones failed to react under the prevailing conditions.

p-*Phenylenediamine reagent.* With aromatic aldehydes, freshly prepared 1% aqueous solution of *p*-phenylenediamine gave yellow to orange colours against a bluish-gray background[12].

Azobenzene-phenylhydrazine sulphonic acid. With aromatic aldehydes 0.5% aqueous solution of azobenzene-phenylhydrazine sulphonic acid upon successive spraying with 1 *N* HCl gave after a few minutes yellow to bluish-green spots on an almost light-brownish background[13].

Pyridine and acetic anhydride reagent. This reagent was made up of 7 parts by volume of pyridine and 3 parts by volume of acetic anhydride, according to GODIN[14]. It was found useful in distinguishing between some aliphatic dicarboxylic acids. With the compounds listed in Tables 18–21, the following colours were obtained with this reagent:

(1) In U.V. only:

glyceric, mesoxalic and β-ketoglutaric acids	light blue
citric acid, ketipic acid diethyl ester	blue-green
dl-tartaric acid	green

(2) Visible:

oxalacetic acid, itaconic acid	red-violet
fumaric acid	yellow
trans- and *cis*-aconitic acid, acetylenedicarboxylic acid	brown

Cyanoacetic acid ester reagent for 1,4-quinones[15]. 1 ml cyanoacetic acid ethyl ester in 14 ml ethanol was sprayed. On subsequent spraying with dilute ammonia the colours obtained with the following compounds were:

thymoquinone, 2,6-xyloquinone, 1,4-benzoquinone	blue
1,4-naphthoquinone, 2-methyl-1,4-naphthoquinone	violet
3-hydroxy-2-methyl-1,4-naphthoquinone	red

For other reagents, which might in some special cases be useful, the reader is referred to the comprehensive treatise by LEDERER AND LEDERER[16].

6. THE CHROMATOGRAPHIC APPARATUS

The chromatographic apparatus consists of six rectangular glass jars (20 × 30 cm and 60 cm high), for six different solvent systems, designated as A, B, C, D, E, F. Each jar is provided with two glass troughs, each of which takes two chromatographic papers, 27 × 55 cm. However, sheets of Whatman No. 1 filter paper 24 × 55 cm were used throughout the experiments. These were cut from large sheets of Whatman paper, the downward flow of solvent being parallel to the machine-direction of the paper.

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The troughs were supported and kept fixed in the right position by means of a Pyrex glass-rod frame as shown in Fig. 4.

For the solvents A, B, C and D a smaller glass dish, about 5 cm deep, containing the water phase of the solvent is placed at the bottom of the jar. In order to keep the atmosphere in the jar saturated with water, two strips of thick paper, approx. 18 cm wide, fixed under the respective troughs, were hung up so as to reach the bottom of

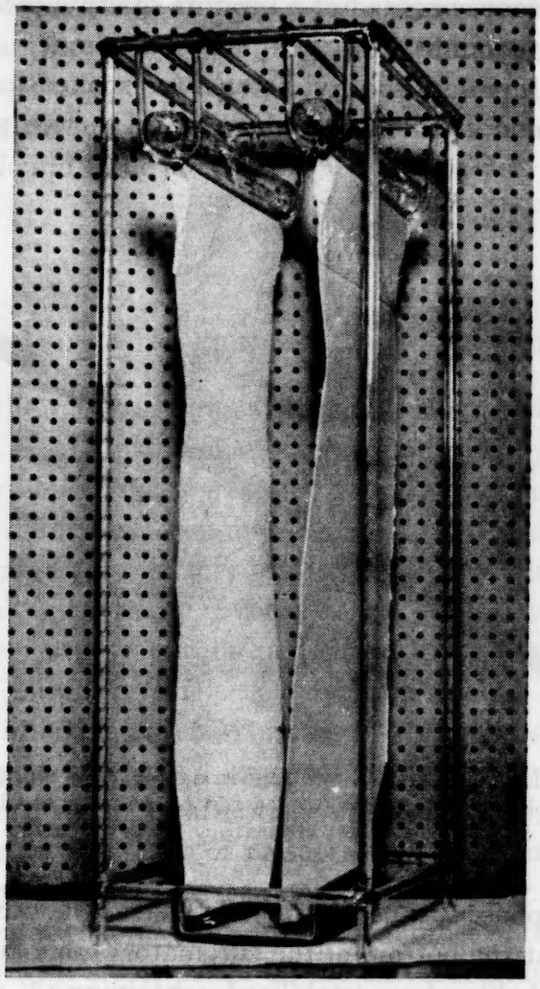

Fig. 4. Pyrex glass frame inside the chromatographic jars. The bottom solution containing the water phase is placed in a rectangular dish.

the dish. The jar was lined from the bottom to a height of about 10 cm with a strip of thick paper. This was necessary in order to maintain saturation with the organic solvent. The organic solvent covered the bottom of the jar (around the dish) to a height of approx. 1 cm. If, after some time, the level rose to about 4–5 cm the solvent in the jar was entirely replaced. The top of the jar was covered by a glass plate, which was ground so that it fitted tightly; no grease was required. The jars were placed in a constant temperature-controlled room at 21°. The solvents were prepared in 10-l portions, which kept well; they were stored in bottles provided with stopcocks, through which the solvents were poured into the troughs.

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7. CHROMATOGRAPHIC SOLVENTS

Six different solvent systems were used in the investigation of reference substances and metabolic products from *Penicillium* series. These solvents were originally composed by HÖGSTRÖM[17] for that purpose.

Solvent F. The solvent consisted of a mixture of ethyl methyl ketone 80 parts, acetone 4 parts, water 12 parts and formic acid (100%) 2 parts by volume.

Solvent E. The solvent consisted of a mixture of ethyl methyl ketone 921 parts, water 77 parts and diethylamine (100%) 2 parts by volume.

Solvent A. 1000 ml methyl isobutyl ketone was shaken for 1 h with 100 ml 4% formic acid (4 ml formic acid and 96 ml water). The two phases were separated

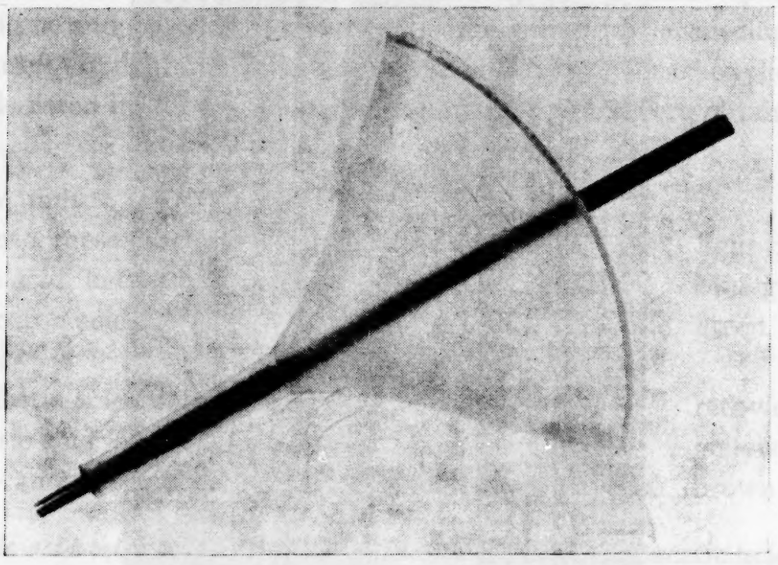

Fig. 5. "Pen" for application of substance solutions in narrow lines on chromatographic paper. The "pen" is made from a polyethylene funnel and a pin of stainless steel and has been devised by Dr. S. LYBING in this laboratory. (Magnification 2×.)

and the clear organic phase was used as mobile phase. The water phase was placed in a separate container at the bottom of the chromatographic jar.

Solvent B. 1000 ml chloroform (stabilized, containing 1% ethanol) was shaken for 1 h with 200 ml solvent of the following composition: 100 parts methanol, 96 parts water and 4 parts by volume formic acid. After separation, the organic phase was used as mobile phase and the water phase was placed in a separate container at the bottom of the jar.

Solvent C. A mixture of 900 ml benzene and 100 ml ethyl methyl ketone was shaken for 1 h with 100 ml 2% formic acid (2 ml formic acid and 98 ml water). After separation, the organic phase was used as mobile phase and the water phase was placed in a separate container at the bottom of the jar.

Solvent D. 1000 ml benzene was shaken for 1 h with 100 ml 2% formic acid. After separation, the organic phase was used as mobile phase and the water phase was placed in a separate container at the bottom of the jar.

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All solvents used were C.P. grade and no extra purification was found to be necessary. In the case of both formic acid and diethylamine 100% products were used. Distilled water was used in all preparations.

Usually 10 l of each solvent were prepared. The solvents were stored tightly closed for 9 months and in our experience storing does not influence their developing power in chromatographic separation. However, especially when using systems B, C, D and E, it was found necessary to change the bottom solvent in the chromatographic jars, as well as the solvent in the troughs every second month. The temperature in the room where the jars were placed was kept as close to 21° as possible by means of thermostatic control.

After mixing the solvents with equal amounts of 90% aqueous ethanol, the pH for each solvent was found to be as follows:

Solvent	F	E	A	B	C	D
pH value	2.7	10.0	3.9	4.8	4.3	5.8

8. COLOUR ESTIMATION AND RECORDING OF DATA

Colour recording of the chromatograms was performed according to an arbitrary standard colour chart, in which the colours were numbered in 72 shades. This followed the "Derwent" colour pencils index made by Cumberland Pencil Co. Ltd. England. Other colour charts were found to be more complicated to handle for everyday use. These colour shades proved satisfactory for describing the colour reactions employed in this investigation, although many of them were not used owing to the fact that the difference between them was practically indistinguishable.

For comparative purposes, single colours were applied with colour pencils as spots, about one inch in diameter, on Whatman No. 1 paper strips. Within the same spot the colour was applied in such a way that its intensity varied. The best results were obtained by rubbing the spots, after they had been drawn, with cotton moistened with carbon tetrachloride. Six series of 12 spots each were numbered and the whole was fixed between acrylate plates, as seen on the right in Fig. 1.

After each spraying with a reagent, the index number of the colour produced was immediately indicated with a soft pencil on the chromatographic spot. At the same time the spot was encircled. In some cases when the colours altered quickly, within a few seconds, from one definite colour to another, two numbers were indicated in the spot. An immediate evaluation was necessary, since most of the colours produced by the action of different reagents were unstable or were changed by the vapours of other reagents in the neighbourhood. As previously stated, the colours were read off against an illuminated screen. The main difficulty was to avoid subjective evaluation of colours. It is also important not to neglect errors caused by variations in the concentrations of the substances, which influence the colour intensity, or the possible overlapping of another substance or impurity. However, the colour reactions recorded in Tables 1–21, obtained with approx. 25 γ of substance on an area of 10 × 20 mm, indicate rather good agreement of the colour pattern with that of the structural

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analogues of the same substance. Even if they are recorded as one of the numbers of a sequence, for instance 30, 31 or 32, 33, this does in practice refer to the same colour. This variation in colour shades occurred during the investigation of reference substances, when using on purpose a special numerical code instead of the chemical nomenclature in designating the substances during spraying, in order to avoid subjective colour estimation. At the same time various types of compounds were investigated in order to obtain reliable information concerning the reagents used. Slight deviations in the indicated colours, especially when diazonium reagents were used, as compared later with those of structural analogues, demonstrated the limits of the probable experimental error.

It should be pointed out that in many cases, well-known colour reactions for

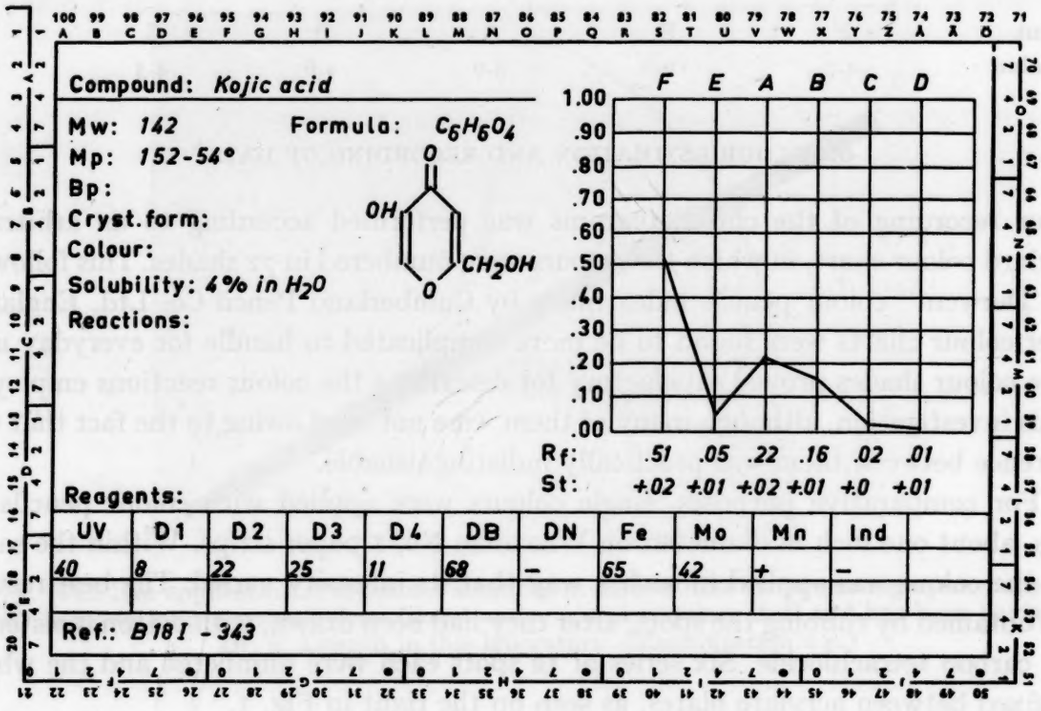

Fig. 6. Sample of record cards for the reference substances.

certain compounds could not be detected with the concentrations used on the paper (for instance ferric chloride produces a colour with resorcinol in a test tube experiment, but here the result was negative). On the other hand, some unusual reactions may be due to impurities, which give mixed colours when they overlap the substances. In these cases, where the impurities had distinctly different R_F values, they could easily be overlooked. Another effect, which is mainly caused by the rate of the reaction, has been observed when spraying the same substance from six different solvent systems. This is obviously connected with the different amounts of acid or alkali remaining in the paper. The colour pattern turns out almost the same, but develops at a different speed.

When the total amount of substance was smaller than about 200 μg, the colour

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reactions were first tested as spots on a filter paper and the R_F values were afterwards estimated by developing one spot for each solvent system with the best detection reagent.

The colour in ultraviolet light was estimated before spraying, the same colour index being used, and the fluorescent regions were drawn by pencil. However, the accuracy was much less when dealing with strongly fluorescent substances, the brightness of which varied with the pH of the filter paper. Some additional information was obtained when after spraying, the sprayed areas were inspected in U.V. These colour-shifts were not included in the Tables.

Single substances of the reference system were recorded on special punched cards, as shown in Fig. 6. The R_F values were entered on the R_F-network as a diagram. The colours were recorded by numbers on a special section of the cards and were also indicated by means of the colour pencils. A complete record of the numerical data obtained from mixtures, which data consisted of R_F values and of a numbered code of colour reactions, was made with a typewriter on special forms of 22 × 36 cm size. Each form was divided vertically into 6 parts corresponding to the solvent systems used. Each part was divided in turn into 12 vertical columns for the reagents used. Different R_F levels were indicated horizontally. The code-numbered colour reactions were entered horizontally on the forms alongside the appropriate R_F values, representing the colour-reaction sequence for a single substance (or several substances in the case of overlapping). By connecting the middle of the six similar colour sequences, a diagram is obtained, which can be redrawn on the same form on a smaller scale on the R_F-network for comparative purposes. This smaller diagram (of which six on each form were reserved for that purpose) corresponds in size to the R_F-network used for recording the compounds on the special cards of the reference system and can be compared directly as to the type of diagram and mean colour sequence.

When all components of the mixture give almost the same colour pattern, which is often the case when homologous compounds are present, it is sometimes advisable to number the different spots uniformly on 6 chromatograms which appear to belong together. Evaluation is facilitated when the spot areas are connected after the spraying is finished, so that a diagram is obtained. When freshly sprayed, the different spots can be more easily distinguished as to their reactivity and their relative differences in size. Also rapid shifts of colour shades could in many cases be of help in localizing the R_F-position of one and the same substance.

9. GUIDE TO TABLES 1–21

The following Tables present information compiled for approx. 450 organic compounds, investigated by the procedure outlined in the previous sections. The R_F values are recorded from six different solvent systems designated by F, E, A, B, C and D. For the composition of these solvents, see the list of abbreviations given below. Under the heading "Detection" (columns 2–11) the colour reactions are recorded for 10 different reagents used for the identification of each compound; in addition, the colour produced in ultraviolet light is indicated in the first column under this heading. The amount of substance used in these experiments was 25–50 γ per spot, which produced different colours when the reagents were applied to them. These colours are referred to by numbers, the explanation of which is found in the colour index. For abbreviations of the reagents, the reader is referred to the following section.

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Table 1 Monohydric phenols and their derivatives.
Table 2 1,2-Dihydric phenols and their derivatives.
Table 3 1,3-Dihydric phenols and their derivatives.
Table 4 1,4-Dihydric phenols and their derivatives.
Table 5 1,2,3- and 1,3,5-Trihydric phenols, derivatives and related compounds.
Table 6 Naphthalene derivatives.
Table 7 Benzoic acid derivatives.
Table 8 Aromatic non-phenolic monocarboxylic acids with the COOH group in the side chain and their derivatives.
Table 9 Aromatic non-hydroxylated di-, tri- and tetracarboxylic acids, and their derivatives.
Table 10 Aromatic and heterocyclic amino acids.
Table 11 Biologically active nitrogen compounds, *e.g.* biogenic amines, etc.
Table 12 Coumarin derivatives.
Table 13 1,2- and 1,4-Pyrone derivatives.
Table 14 Naturally occurring tropolones.
Table 15 Natural products of vegetable origin, *e.g.* pinosylvin, hesperetin, quercetin, etc.
Table 16 Miscellaneous compounds.
Table 17 Metabolic products from moulds and *Penicillium* species.
Table 18 Unsaturated aliphatic mono-, di- and tricarboxylic acids.
Table 19 Aliphatic dicarboxylic acids.
Table 20 Aliphatic hydroxy acids.
Table 21 Aliphatic keto acids and hydroxy- and keto-compounds of biological interest.

ABBREVIATIONS USED IN TABLES 1–24

Chromatographic solvent systems:

F = Ethyl methyl ketone–acetone–formic acid–water
E = Water–ethyl methyl ketone–diethylamine
A = Methyl isobutyl ketone–formic acid–water
B = Chloroform–methanol–formic acid–water
C = Benzene–ethyl methyl ketone–formic acid–water
D = Benzene–formic acid–water

For preparation of the different solvents see the section on *Chromatographic solvents*.

Reagents used for detection:

U.V. = Ultraviolet light
D1 = Diazotized sulfanilic acid
D2 = Diazotized 4-benzoylamino-2,5-dimethoxyaniline
D3 = Diazotized *o*-dianisidine
D4 = *p*-Nitrobenzenediazonium fluoroborate
DB = 2,6-Dibromoquinone-4-chloroimide
DN = 2,4-Dinitrophenylhydrazine
Fe = Ferric chloride
Mo = Phosphomolybdic acid
Mn = Potassium permanganate
Ind = Bromophenol blue

For preparation of the various reagents see the section on *Spraying reagents*.

Owing to the lack of space in the Tables, the R_F values have been recorded as 12, 56, 88 but should be read: 0.12, 0.56, 0.88 etc. The colours produced by the reagent are recorded as numbers, and the corresponding shades can be found in the colour index. The — sign means that no reaction was observed under the prevailing conditions. The + sign indicates an uncertain reaction, which was too weak to deserve colour estimation. Reactions with the reagents Mn and Ind are only indicated by the signs: —, + or ++. The ++ sign means that a positive reaction was obtained immediately. In the few cases where the colours are recorded by a number placed on top of another number, *e.g.* $\left.\begin{matrix}24\\57\end{matrix}\right\}$, this indicates that immediately upon spraying, a violet spot (24 in the colour index) appears which, within a few seconds, turns to light brown (57 in the colour index). Usually most colours are unstable and after some time take on a brownish tone, which is to some extent caused by the chemical influence of other reagents used in the vicinity. This change in colour is not recorded in the tables, nor is there any column for those compounds that, at this low concentration, are visible on the chromatograms because of their own colour.

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COLOUR INDEX FOR THE TABLES

The colours produced by the action of different reagents on the investigated compounds, presented in Tables 1–24, have been recorded by number, according to the following code:

01 Zinc Yellow	25 Dark Violet	49 Sap Green
02 Lemon Cadmium	26 Light Violet	50 Cedar Green
03 Gold	27 Blue Violet Lake	51 Olive Green
04 Primrose Yellow	28 Delft Blue	52 Bronze
05 Straw Yellow	29 Ultramarine	53 Sepia
06 Deep Cadmium	30 Smalt Blue	54 Burnt Umber
07 Naples Yellow	31 Cobalt Blue	55 Vandyke Brown
08 Middle Chrome	32 Spectrum Blue	56 Raw Umber
09 Deep Chrome	33 Light Blue	57 Brown Ochre
10 Orange Chrome	34 Sky Blue	58 Raw Sienna
11 Spectrum Orange	35 Prussian Blue	59 Golden Brown
12 Scarlet Lake	36 Indigo	60 Burnt Yellow Ochre
13 Pale Vermilion	37 Oriental Blue	61 Copper Beech
14 Deep Vermilion	38 Kingfisher Blue	62 Burnt Sienna
15 Geranium Lake	39 Turquoise Blue	63 Venetian Red
16 Flesh Pink	40 Turquoise Green	64 Terra Cotta
17 Pink Madder Lake	41 Jade Green	65 Burnt Carmine
18 Rose Pink	42 Juniper Green	66 Chocolate
19 Madder Carmine	43 Bottle Green	67 Ivory Black
20 Crimson Lake	44 Water Green	68 Blue Grey
21 Rose Madder Lake	45 Mineral Green	69 Gunmetal
22 Magenta	46 Emerald Green	70 French Grey
23 Imperial Purple	47 Grass Green	71 Silver Grey
24 Red Violet Lake	48 May Green	72 White=colourless

TABLE 1

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SOME MONOHYDRIC PHENOLS AND THEIR DERIVATIVES

R_F values × 100						Compounds	Detection									
F	*E*	*A*	*B*	*C*	*D*		*U.V.*	*D1*	*D2*	*D3*	*D4*	*DB*	*DN*	*Fe*	*Mo*	*Mn*	*Ind*
91	95	93	91	92	92	Phenol	—	05	08	08	60	38	—	—	—	+	—
90	93	88	93	92	90	Anisole	—	08	14	21	08	32	—	—	—	+	—
93	95	98	97	93	88	*o*-Cresol	—	—	06	—	08	38	—	—	—	+	—
94	94	97	96	93	88	*m*-Cresol	—	05	06	11	08	38	—	—	—	+	—
94	95	96	96	92	87	*p*-Cresol	—	—	06	06	08	72	—	—	—	+	—
95	90	91	93	90	90	4-Chloro-*m*-cresol	33	08	13	60	08	32	—	—	—	+	—
93	91	92	92	92	90	2,3,6-Trichloro-*p*-cresol	—	06	60	17	57	69	—	—	71	++	+
90	88	95	89	92	92	2,3-Xylenol	—	08	10	15 / 08	08	25	—	—	30	++	—
93	95	94	96	96	88	2,4-Xylenol	—	—	64	64	08	—	—	—	30	+	—
94	94	92	97	96	90	2,5-Xylenol	—	57	06	08	60	30	—	—	30	+	—
95	94	92	97	96	91	2,6-Xylenol	—	—	07	21	—	28	—	—	40	—	—
91	94	91	95	94	89	3,4-Xylenol	—	—	06	—	—	—	—	—	30	—	—
88	93	93	94	93	88	3,5-Xylenol	—	—	07	—	08	67	—	—	—	+	—
89	92	97	96	93	91	2,3,5-Trimethylphenol	—	—	07	08	64	28	—	—	30	+	—
88	86	93	95	93	92	2,4,5-Trimethylphenol	—	07	07	61	64	—	—	—	—	++	—
92	90	97	90	92	89	2-Propenylphenol	—	—	—	07	09	38	—	—	30	+	—
95	91	91	94	93	92	4-*tert.*-Butylphenol	—	—	10	19	—	31	—	—	30	++	—
94	91	94	95	94	94	4-*tert.*-Octylphenol	—	—	10	19	—	—	—	—	—	+	—
94	96	84	93	92	92	Carvacrol	—	07	08	09	08	33	—	—	30	++	—
90	92	97	96	93	92	Thymol	—	—	07	08	60	24	—	—	30	+	—
93	93	86	94	92	92	3-Methyl-4-*tert.*-butylphenol	—	—	07	09	06	25	—	—	—	++	—
50	83	42	29	06	00	*o*-Aminophenol	—	06	58	58	64	35	60	63	57	++	—
75	91	76	40	12	06	*m*-Aminophenol	71	11	15	65	63	25	—	52	68	++	—
25	72	07	06	00	00	*p*-Aminophenol	—	27	—	—	66	69	—	25	52	++	—
92	68	92	88	87	88	*o*-Nitrophenol	65	06	—	—	62	—	—	—	—	+	—

(Contd. on p. 56)

TABLE 1 *(continued)*

R_F values × 100						Compounds	Detection										
F	*E*	*A*	*B*	*C*	*D*		*U.V.*	*D1*	*D2*	*D3*	*D4*	*DB*	*DN*	*Fe*	*Mo*	*Mn*	*Ind*
93	86	88	89	89	67	*m*-Nitrophenol	65	06	—	—	09	—	—	—	—	+	—
92	65	87	88	83	47	*p*-Nitrophenol	65	—	—	—	60	—	—	26	—	+	—
92	67	88	95	92	91	2,4-Dinitrophenol	65	—	—	—	60	—	72	—	—	+	++
97	64	92	93	93	92	2,5-Dinitrophenol	65	—	—	—	—	—	72	57	—	+	+
94	74	91	93	95	93	2,6-Dinitrophenol	65	—	—	—	60	—	72	—	—	+	++
76	83	34	13	36	30	Picric acid	65	—	—	—	—	—	—	—	—	+	++
94	73	93	94	92	93	4,6-Dinitro-*o*-cresol	65	—	—	—	—	—	—	—	—	+	—
91	69	93	92	92	78	6-Nitro-*m*-cresol	53	—	—	52	10	—	72	57	48	+	—
82	86	80	29	86	76	2,4,6-Trinitro-*m*-cresol	65	—	—	57	—	—	72	—	—	+	++
85	90	78	66	58	24	Saligenin	—	03	13	13	09	33	—	26	—	+	—
84	90	91	80	70	51	5-Methylsaligenin	—	—	—	06	11	—	—	30	30	+	—
84	83	89	90	85	60	2-Hydroxyacetophenone	—	15	—	62	13	38	06	—	—	++	—
89	89	83	90	89	71	3-Hydroxyacetophenone	—	06	60	64	12	39	03	24	—	+	—
87	76	87	87	81	42	4-Hydroxyacetophenone	—	—	—	—	13	—	07	—	—	+	—
91	82	89	88	86	58	4-Hydroxypropiophenone	—	07	—	—	09	—	05	—	—	+	—
93	47	90	84	90	64	Salicylaldehyde	33	06	03	06	—	38	06	25	—	+	—
94	92	87	88	83	55	3-Hydroxybenzaldehyde	—	—	—	—	—	38	06	—	—	+	—
94	69	85	85	80	37	4-Hydroxybenzaldehyde	—	03	—	63	—	—	08	—	—	+	—
90	90	93	88	93	89	Anisaldehyde	—	—	—	—	—	32	03	—	—	+	—
94	92	93	93	90	88	3-Methylsalicylaldehyde	—	09	12	65	10	43	06	25	—	+	—
92	51	92	87	86	52	Salicylic acid	33	06	—	—	07	—	—	24	—	+	+
92	11	92	78	88	33	Thiosalicylic acid	33	01	07	07	08	12 / 21	—	58	30	++	+
92	97	91	83	59	17	1,1-Hexahydrophenolcarboxylic acid	—	—	—	—	—	—	—	—	—	—	+
92	38	88	73	79	50	O-Carbomethoxysalicylic acid	34	—	—	—	06	—	—	—	—	—	+
90	37	84	83	92	64	3-Chloroacetylsalicylic acid	33	—	—	—	08	33	—	24	—	+	++
94	67	86	87	85	70	5-Chlorosalicylic acid	33	—	—	—	08	—	—	25	—	+	++
91	09	86	45	35	01	3-Hydroxybenzoic acid	—	06	—	65 / 58	07	33	—	—	—	+	+
80	35	72	37	11	00	2-Hydroxymethyl-3-hydroxybenzoic acid	33	08	—	65	12	34	—	+	—	+	+
80	64	72	62	37	08	4-Hydroxyphthalide	33	03	—	64	58	—	—	24	—	+	—
88	08	91	37	28	01	4-Hydroxybenzoic acid	—	06	—	65 / 58	07	—	—	03	—	+	+
93	93	92	89	87	64	4-Hydroxybenzoic acid methyl ester	—	—	—	—	—	—	—	—	—	+	—
92	13	93	92	87	74	Anisic acid	—	—	—	—	07	—	—	03	—	—	+
93	11	92	39	43	05	4-Aminosalicylic acid	34	11	12	63	12	26	—	65	—	++	+
41	21	09	00	00	00	5-Aminosalicylic acid	48	—	62	64	03	53	—	65	68	++	++
73	68	45	05	15	03	3,5-Dinitrosalicylic acid	69	—	—	—	08	—	—	59	—	—	++
83	03	77	20	43	03	3,5-Dinitro-4-hydroxybenzoic acid	65	—	—	—	—	—	—	—	—	+	++
92	20	93	89	88	70	*o*-Cresoxyacetic acid	—	—	—	—	—	—	—	—	—	—	++
92	18	90	92	84	59	*m*-Cresoxyacetic acid	—	—	—	—	—	—	—	—	—	—	++
91	19	88	92	84	59	*p*-Cresoxyacetic acid	—	—	—	—	—	—	—	—	—	—	++
94	61	92	92	92	72	3-Methylsalicylic acid	33	06	—	—	07	33	—	24	—	+	+
94	55	92	91	90	67	4-Methylsalicylic acid	33	06	—	—	08	33	—	23	—	+	+
91	46	89	90	89	65	5-Methylsalicylic acid	38	—	—	—	—	—	—	25	—	++	+
88	62	78	90	85	68	6-Methylsalicylic acid	33	08	08	62	09	35	—	23	—	+	+
89	10	87	91	82	74	3-Methoxyphenylacetic acid	—	—	—	—	57	—	—	—	—	—	+
87	08	87	91	82	74	4-Methoxyphenylacetic acid	—	—	—	—	—	—	—	—	—	—	+
91	08	88	58	50	05	4-Hydroxyhydrocinnamic acid	—	—	—	—	—	—	—	—	—	+	+
94	18	94	68	52	00	*o*-Coumaric acid	33	08	11	65 / 07	07	41 / 35	03	03	32	++	+
90	94	96	92	88	58	*o*-Coumaric acid methyl ester	33	06	07	09	62	41	—	—	60	++	—
90	27	94	50	74	25	4-Hydroxyphenylpyruvic acid	33	—	06	25	08	50	06	—	—	++	+
92	03	80	07	04	00	5-Hydroxy-3-indoleacetic acid	03	14	65	25	62	68	—	—	30	++	+
83	00	60	08	05	00	3-Hydroxyphthalic acid	33	—	—	57	09	35	—	65	—	+	++
68	04	62	12	07	00	2-Hydroxyisophthalic acid	33	—	—	—	08	—	—	64	—	—	+
86	02	92	24	13	00	4-Hydroxyisophthalic acid	33	—	—	—	—	—	—	65	—	—	++

TABLE 2

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SOME 1,2-DIHYDRIC PHENOLS AND THEIR DERIVATIVES

R_F values × 100						Compounds	Detection										
F	E	A	B	C	D		U.V.	D1	D2	D3	D4	DB	DN	Fe	Mo	Mn	Ind
88	88	86	57	55	10	Pyrocatechol	—	17	70	—	63	35	50	50 67	57	+	—
90	93	89	94	92	91	Guaiacol	—	08	14	22	08	35	—	—	68	++	—
89	93	94	94	92	92	Veratrole	—	08	14	21	—	32	—	—	—	+	—
92	79	92	80	79	35	3-Methylpyrocatechol	—	71	17	18	12	35	50	54	57	+	—
93	91	95	82	76	32	4-Methylpyrocatechol	—	12	24	21	62	35	71	52	60	+	—
91	92	87	93	95	91	4-Methylguaiacol	—	17	10	62	24	68	—	58	30	++	—
91	92	93	95	93	95	Eugenol	—	—	10	56	64	42 09	—	—	30	++	—
89	91	96	96	94	91	Isoeugenol	—	—	—	—	60	07	—	—	30	++	—
92	89	88	95	92	93	Isoeugenol acetate	—	05	—	—	—	—	—	—	—	+	—
77	69	72	57	32	17	Vanillyl alcohol	—	—	12	22 12	11	35	—	—	30	+	—
83	77	83	80	58	43	*o*-Vanillyl alcohol	—	09	12	65	25	35	58	56	52	++	—
90	85	92	90	86	82	5-Hydroxymethyl-eugenol	—	13	10	13	—	32	03	—	30	+	—
85	81	88	83	74	62	4-Ethylhydroxymethylguaiacol	—	—	13 06	14 08	60	35	—	—	30	+	—
84	67	91	93	92	90	*o*-Vanillin	33	07	10	12	12	41 38	07	68	48	++	—
83	85	87	94	92	89	*o*-Veratraldehyde	—	17	—	—	—	32	07	—	—	+	—
92	71	96	93	92	93	*o*-Vanillin acetate	33	08	10	15	10	38	03	71	03	+	—
86	44	88	48	28	01	Protocatechualdehyde	—	—	—	—	59 64	50	07	43	06	++	—
83	81	94	89	78	67	Isovanillin	33	07	10	12	12	41	07	68	48	++	—
84	44	92	90	84	78	Vanillin	—	—	—	—	62	38	07	—	—	++	—
87	89	88	94	92	92	Veratraldehyde	30	06	03	63	10	33	07	—	—	+	—
86	83	86	84	95	92	Piperonal	—	—	—	—	—	—	07	—	—	+	—
88	23	94	94	89	92	4-Acetoxy-5-methoxyisophthal-aldehyde	48	06	—	—	—	—	03	27	04	+	—
88	73	93	89	82	73	Coniferyl aldehyde	38	07	07	07	—	07 27	—	—	—	+	—
88	86	87	93	94	92	3,4-Dimethoxyacetophenone	—	07	65	21	06	33	06	—	—	+	—
88	01	85	61	38	06	3-Methoxy-4-hydroxy-ω-hydroxy-methyl-acetophenone	34	08	—	24	09 60	33	+	—	71	+	—
88	34	84	39	38	04	2,3-Dihydroxybenzoic acid	32	13	—	—	12	34 05	—	30	68	+	+
93	12	93	92	86	78	*o*-Veratric acid	—	—	—	—	—	—	—	—	—	—	++
84	03	67	05	02	00	Protocatechuic acid	—	13	—	—	59	71	—	42	57	+	+
83	04	89	62	42	08	Vanillic acid	—	08	—	65	07	33	—	08	68	++	+
80	05	94	91	78	67	Veratric acid	—	—	—	—	—	—	—	08	—	—	+
84	07	87	93	83	93	3,4-Dimethoxyphenylacetic acid	—	06	13	14	57	34	—	—	—	+	+
87	04	89	81	61	23	3-Methoxy-4-hydroxyphenylpropionic acid	—	—	—	—	60	27	—	—	—	+	+
78	02	76	08	03	00	Caffeic acid	40	03	22	58	62	42 53	—	42 67	03	++	+
83	02	87	68	41	12	Ferulic acid	33	—	17	25 64	14	25	—	59	40 71	+	+
85	03	85	89	72	49	3,4-Dimethoxycinnamic acid	33	—	—	—	06	—	—	58	—	+	+
93	31	97	92	86	47	2-Methoxy-5-propylphenoxyacetic acid	—	—	—	—	—	—	—	—	—	—	++
92	26	88	92	89	84	5-Methoxy-6-hydroxyisophthal-aldehydic acid	48	—	—	—	—	—	03	—	—	—	+
81	00	81	79	70	23	4-Methoxy-5-hydroxyisophthal-aldehydic acid	33	—	—	—	64	—	06	58	—	+	+
83	00	63	09	06	00	4-Hydroxy-5-methoxyisophthalic acid	30	03	—	—	08	—	—	24	—	—	+

TABLE 3

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SOME 1,3-DIHYDRIC PHENOLS AND THEIR DERIVATIVES

R_F values × 100						Compounds	Detection										
F	E	A	B	C	D		U.V.	D1	D2	D3	D4	DB	DN	Fe	Mo	Mn	Ind
88	91	83	22	28	02	Resorcinol	34	05	15	21	11	65	—	—	—	+	—
88	89	88	18	32	02	*m*-Propoxyphenol	—	08	15	23	12	25	—	—	29	++	—
89	93	89	42	57	08	2-Methylresorcinol	—	05	15	21	12	25	—	—	68	+	—
94	90	91	38	43	05	Orcinol	34	07	15	65	12	26	—	—	68	+	—
88	84	91	60	66	17	4-Ethylresorcinol	—	—	14	22	12	65	—	—	30	++	—
90	91	91	88	88	81	4-Hexylresorcinol = caprokol	—	07	15	23	12	65	—	—	30	++	—
83	79	94	44	59	10	4-Chlororesorcinol	—	06	14	19	12	25	—	—	30	++	—
92	61	91	87	85	42	2,4-Dihydroxybenzaldehyde	—	07	19	22	09	68	07	61	—	++	—
92	73	94	89	90	60	4-Methyl-2,6-dihydroxybenzaldehyde = atranol	65	06	12	65	10	68	03	69	42	+	—
88	62	88	85	83	49	2,4-Dihydroxyacetophenone = resacetophenone	03	03	15	21	09 63	70	+	65	—	+	—
90	82	88	90	90	67	2,6-Dihydroxyacetophenone	—	07 48	19	23	11	25 51	+	70	51	++	—
87	27	83	22	33	01	2,4-Dihydroxybenzoic acid	—	02	15	22	09	24	—	24	68	+	+
94	93	93	94	93	92	2,4-Dimethoxybenzaldehyde	34	06	08	62	—	—	08	—	03	+	—
77	02	77	01	01	00	3,5-Dihydroxybenzoic acid	—	—	15	19	08	25	—	57	25 30	++	+
72	74	61	14	23	04	2,6-Dihydroxybenzoic acid	—	02	15	21	09	29	—	25	—	+	+
93	28	93	47	52	02	Orsellinic acid	—	11	14	22	08	25	—	24	—	++	+
88	81	74	49	68	18	*p*-Orsellinic acid	—	06	19	21	08	24 69	—	28	—	+	+
92	04	83	02	04	00	4-Bromo-3,5-dihydroxybenzoic acid	—	08	19	22	08	28	—	—	30	+	+
89	87	87	83	90	88	2,5-Dimethyl-4,6-dihydroxybenzoic acid methyl ester	—	08	15	21	07	25 51	—	—	51	+	—
92	94	94	93	93	93	2-Methyl-4-methoxy-6-hydroxybenzoic acid methyl ester	—	07	07	23	08	38	—	—	—	—	—
79	00	54	02	00	00	3,5-Dihydroxyphthalic acid	33	07	—	21	10	27	—	65	—	+	+
78	00	62	05	00	00	3,5-Dihydroxy-6-methoxyphthalic acid	33	07	—	19	09	25	—	25 65	01	++	+
79	02	62	79	25	11	Dihydroresorcinol	—	06	15	15	03	56	—	58	71	++	+
86	04	92	82	65	46	5,5-Dimethyldihydroresorcinol	—	—	11	12	01	—	—	62	—	++	+
84	78	84	18	31	02	Hexahydroresorcinol	—	11	14	19	12	25	—	—	30	++	—
92	59	93	95	93	94	2-Nitro-5-methylresorcinol	65	07	19	65	09	69	—	—	—	++	—
94	49	95	91	80	75	4-Nitro-5-methylresorcinol	65	07	19	64	09	69	—	52	—	++	—

10. RESULTS AND DISCUSSION

This investigation is an attempt to systematize common paper-chromatographic analytical methods in order to make it possible to carry out with a reasonable degree of probability, a preliminary identification of the structure of phenolic compounds derived from biological sources, especially those arising from the metabolism of fungi. It has mainly been based on the comparative study of compounds collected into a suitable reference system, in which six R_F values were presented in diagramsrtogether with their corresponding colour-reaction patterns. The values were compared with similar data obtained when unknown extracts were examined.

In all about 450 compounds were investigated with regard to their behaviour towards six chromatographic solvent systems and ten colour reagents. About 2,700

References p. 74.

TABLE 4

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SOME 1,4-DIHYDRIC PHENOLS AND THEIR DERIVATIVES

R_F values × 100						Compounds	Detection										
F	E	A	B	C	D		U.V.	D_1	D_2	D_3	D_4	DB	DN	Fe	Mo	Mn	Ind
87	92	79	15	20	01	Hydroquinone	—	72	51	72	13	58	—	—	42 70	+	—
74	61	26	16	03	00	Hexahydroquinone = quinitol	—	—	—	—	—	—	—	—	71	+	—
94	93	93	91	95	94	*p*-Butyloxyhydroquinone	—	09	62	62	14	35	—	—	28	++	—
89	85	90	35	42	05	Toluhydroquinone	—	56	—	—	11	—	—	—	71	+	—
88	93	94	48	60	16	2,6-Xylohydroquinone	—	—	72	72	—	67	—	—	30	+	—
95	94	97	84	90	72	Thymohydroquinone	—	72	13	72	—	69	—	—	30	++	—
75	02	58	03	02	00	Gentisic alcohol	34	52	56	52	51	70	—	+	42 30	++	—
50	00	15	00	00	00	2,5-Dihydroxymethylhydroquinone	33	—	—	06	08	69	—	—	30	+	—
97	97	95	82	80	42	Gentisic aldehyde	33	—	—	—	60	70	07	28	30	+	—
88	90	87	89	86	58	2,5-Dihydroxyacetophenone	64	05	06	06	08	54	+	70	01	+	—
91	86	94	93	91	93	2-Hydroxy-5-methoxyacetophenone	03	06	06	06	60	—	—	68	50	++	—
83	78	86	15	19	01	*p*-Benzoquinone	—	—	—	—	63	—	—	—	57	++	—
88	88	87	28	39	05	Toluquinone	13	72	72	72	57	69	03	—	30	++	—
91	92	90	93	86	87	2,6-Xyloquinone	70	—	57	—	—	68	—	—	30	++	—
95	93	95	93	92	91	Thymoquinone	70	—	13	—	—	—	—	—	40	++	—
29	00	02	00	00	00	2,3,5,6-Tetrahydroxymethylquinone	—	—	—	—	—	—	—	—	—	+	—*
31	01	05	00	00	00	Nitranilic acid	59	08	—	—	—	—	—	—	40	+	+
93	dec**	86	94	87	88	2-Methyl-3,5,6-trichloroquinone	—	72	72	72	72	24	—	—	70	+	—
88	36	82	19	25	00	Gentisic acid	38	72	—	—	58	70	—	28	68	+	+
64	04	15	00	00	00	3,6-Dihydroxyphthalic acid	38	72	72	72	14 72	53	—	28	30	++	+
89	20	82	10	14	00	2,3-Dicyanohydroquinone	33	72	72	72	72	53	—	29	27	+	—
89	87	93	93	90	88	4,4′-Dihydroxydiphenyl	—	—	—	—	10	—	—	—	—	+	—

* The colour of the compound itself 09.
** Decomposition.

R_F values were recorded, as well as some 4,000 colour reactions. Owing to the great number of data obtained from these records it is not possible to discuss them in detail here, and I have, therefore, confined myself to describing the more marked effects of certain types of compounds on the R_F values and colour reactions.

In order to give an over-all picture of the effect that different substituents in the phenolic nucleus have on the R_F values in different solvent systems, a selection of appropriate data is presented in Table 22 (p. 71).

For most compounds listed the approximate R_F ranges are given regardless of the position of the different substituents in the phenolic nucleus. As can be seen from Table 22, the best separations were obtained with dihydric phenols. For these phenols, a differentiation has been made according to the position of the OH groups: 1,2; 1,3; 1,4. This could serve as a base when taking into account that each additional group, such as alkyl, halogen, aldehyde or acetyl (or ether groups), increases the R_F values to a certain extent as is the case in solvents B, C, D. An additional carboxyl- (the effect of which is included in this Table), hydroxymethyl-, hydroxy- or amino-group decreases the value, especially in solvents E, B, C and D. Since solvent E is

References p. 74.

TABLE 5

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SOME 1,2,3- AND 1,3,5-PHENOLS, DERIVATIVES AND RELATED COMPOUNDS

R_F values × 100						Compounds	Detection									
F	E	A	B	C	D		U.V.	D1	D2	D3	D4	DB	DN	Fe	Mo	Mn	Ind
82	00	67	08	05	00	Pyrogallol	—	58	65	65	10	60	55	57	57	+	—
86	92	95	95	87	88	1,3-Dimethoxypyrogallol	—	19	21	24	12	35	—	62	70	++	—
88	00	87	44	48	05	4-Ethylpyrogallol	66	08	64	64	06	—	—	70	68	+	—
82	65	60	01	01	00	Phloroglucinol	—	02	65	65	12	70	—	—	68	+	—
87	92	95	95	88	87	Trimethoxyphloroglucinol	—	—	—	—	12	57	—	—	—	—	—
81	00	66	07	07	00	2,4,5-Trihydroxytoluene	—	17	64	64	—	—	57	69	31 25	+	—
88	90	85	94	93	92	Trimethoxygallic aldehyde	—	—	—	—	07	—	06	—	—	+	—
88	31	95	35	32	00	Phloroglucinol aldehyde	—	07	64	65	11	66	07	66	—	++	—
84	89	86	95	80	71	Trimethoxyphloroglucinol aldehyde	33	—	—	—	—	—	07	—	—	—	—
79	07	84	67	48	19	Gallacetophenone	—	06	13	12	06	52	+	52	51	++	—
88	68	96	51	39	00	Acetylphloroglucinol	—	03	65	21	12	69	+	24	30	+	—
77	03	80	04	03	00	Pyrogallol-4-carboxylic acid	34	24	25	25	08	62	—	63	59	++	+
73	00	38	00	00	00	Gallic acid	—	09 70	20	65	09	52	—	68	03 10	+	+
82	02	78	73	29	05	Syringic acid	—	19	19	22	12	35	—	57	68	++	+
81	09	96	92	84	77	Trimethoxygallic acid	—	19	—	—	—	—	—	08	—	+	+
86	87	93	87	92	85	Trimethoxygallic acid methyl ester	—	06	61	62	06	70	—	—	—	—	—
83	04	82	57	34	07	5-Hydroxytoluquinone	—	—	22	22	60	—	03	—	—	++	—
90	00	38	00	00	00	*m*-Digallic acid	—	—	19	17	08	03	—	68	05	++	+
24	00	02	00	00	00	Shikimic acid	—	—	—	—	—	—	—	—	—	+	+
08	09	00	00	00	00	*d*-Quinic acid	—	—	—	—	—	—	—	—	—	+	+

basic, the presence of a carboxyl group in any structure is demonstrated by a considerable lowering of the R_F value in this solvent. This low value is significant for carboxyl groups and is confirmed by a positive reaction with the indicator reagent. Biogenic amines, coumarins and pyrones could also have relatively low R_F values in solvent E, but they gave negative reactions with the indicator reagent.

For simple monohydric phenols, especially with additional alkyl, halogen or ether groups, the resolving power of the solvents was poor, as was to be expected considering the solvents used. The solvents were originally evolved for investigations of metabolic products from *Penicillium* species, but were later extended to cover a large group of other organic compounds of biochemical interest. In this particular case it was sometimes an advantage to separate rapidly the simple phenols, which have high R_F values in all solvents, from compounds of the phenolic carboxylic acid type. In fact, the latter type predominates in products of mould metabolism. For other metabolic compounds found in moulds (including tetronic acids, pyrones, tropolonic acids, compounds from the TCA-cycle and those with more complex structures, for instance erdin and others), the solvents have proved to give satisfactory resolution, at least solvents E, B and C have with mixtures of up to 10 components. However, interpretation in such cases was rather difficult, but was somewhat facilitated when the R_F data were combined with the results of the estimation of the colour reactions with ten different reagents.

References p. 74.

TABLE 6

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SOME NAPHTHALENE DERIVATIVES

R_F values × 100						Compounds	Detection										
F	E	A	B	C	D		U.V.	D1	D2	D3	D4	DB	DN	Fe	Mo	Mn	Ind
90	92	89	94	90	93	2-Acetonaphthone	33	—	—	—	—	03	03	—	—	—	—
92	18	93	92	88	85	α-Naphthylacetic acid	57	—	—	—	—	—	—	—	—	—	+
92	90	95	94	92	88	1-Naphthol	—	13	22	25	19	36 25	—	13 26	27	++	—
92	52	95	87	90	68	1-Hydroxy-2-naphthoic acid	32	09	22 64	65	63	35	—	68	24 71	++	++
92	91	94	93	92	84	2-Naphthol	—	—	22	25	15	43	—	—	27	++	—
94	49	92	79	88	49	2-Hydroxy-3-naphthoic acid	01	12	25	25 35	15	70	—	28	71	++	++
94	94	92	93	92	91	1,2-Naphthoquinone	13	—	—	—	—	—	—	—	—	+	—
93	82	93	56	63	06	1,3-Dihydroxynaphthalene	—	08	19	23	64	53	—	07	08	++	—
92	92	93	58	81	17	1,4-Dihydroxynaphthalene	33	08	23	23	25	68	—	28	28	++	—
92	92	88	91	92	92	1,4-Naphthoquinone	65	13	52	26	15	—	—	—	27	++	—
91	19	93	93	91	84	2-Hydroxy-1,4-naphthoquinone	63	62	—	62	66	—	01	—	—	+	—
93	93	95	94	96	94	2-Methyl-1,4-naphthoquinone	65	03	—	—	—	—	—	—	34	++	—
92	33	85	91	83	87	2-Methyl-3-hydroxy-1,4-naphthoquinone	70	+	+	+	+	—	—	60	+	+	—
86	89	89	52	75	12	1,5-Dihydroxynaphthalene	33	24 15	25	25	12	25	—	—	66	++	—
87	88	89	47	66	11	1,6-Dihydroxynaphthalene	69	12	25	25	11	25	—	65	25	++	—
95	92	94	54	69	10	1,7-Dihydroxynaphthalene	32	15	65 25	24 35	62	35	—	25 28	45 68	++	—
94	92	94	37	60	05	2,6-Dihydroxynaphthalene	32	14	63 25	25 28	65	45	—	48	45 68	++	—
92	88	89	39	45	03	2,7-Dihydroxynaphthalene	—	—	23	25	19	67	—	—	43 68	++	—

Unsuccessful separation of simple monohydric phenols could, to some extent, be compensated by carefully studying the colour-reaction patterns, since these are rather informative when it comes to discriminating between several possible structures. The simple phenols also proved useful for the study of miscellaneous colour reactions when correlating structural characteristics with the colour produced by different reagents. On introducing a hydroxymethyl-, aldehyde-, nitro- or acetyl-group into a simple phenol, the R_F values in solvents C and D decrease to a different extent. If an additional carboxyl group is present, the R_F values fall significantly in solvents E, B, C and D. An amino group causes a similar lowering of the R_F values in solvents B, C and D, but in solvent E the R_F remains more or less the same. A simultaneous decrease in solvents F and A should also be interpreted as significant for the presence of an amino group.

At the bottom of Table 22 (p. 71), several types of aliphatic carboxylic acids are presented. It is interesting to note the wide range of R_F values for solvents F and A, which does not occur when aromatic carboxylic acids are involved. This indicates that when acidic components are found at these R_F levels they very probably belong to an aliphatic series.

In Table 23 (p. 72), a series is collected of those compounds that were available,

References p. 74.

TABLE 7

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SOME BENZOIC ACID DERIVATIVES

R_F values × 100						Compounds	Detection						Remarks
F	E	A	B	C	D		U.V.	D_4	DN	Fe	Mn	Ind	
88	20	98	92	87	79	Benzoic acid	—	—	—	—	—	+	
95	93	94	92	93	92	Hexahydrobenzoic acid	—	—	—	—	—	+	
90	90	94	92	92	94	Acetophenone	—	—	+	—	—	—	
96	90	97	94	94	93	Benzaldehyde	—	07	06	—	+	—	
88	28	85	88	86	80	*o*-Chlorobenzoic acid	—	—	—	—	—	++	
90	39	95	92	92	91	*m*-Chlorobenzoic acid	—	—	—	—	—	++	
94	92	95	94	93	93	*m*-Chlorobenzaldehyde	—	—	06	—	+	—	Mo-71
94	37	91	92	89	92	*p*-Chlorobenzoic acid	—	—	—	—	—	+	
90	46	95	92	92	84	3,4-Dichlorobenzoic acid	—	—	—	—	—	+	
96	16	88	90	80	29	*o*-Nitrobenzoic acid	70	—	—	—	—	+	
96	45	94	88	85	76	*m*-Nitrobenzoic acid	65	—	—	—	—	++	
95	90	94	93	94	92	*m*-Nitrobenzoic acid methyl ester	65	—	—	—	—	—	Mo-71
91	90	91	89	88	86	*m*-Nitrobenzaldehyde	65	—	06	—	+	—	
96	22	92	88	88	88	*p*-Nitrobenzoic acid	70	—	—	—	—	++	
92	63	92	84	82	26	2-Chloro-3,5-dinitrobenzoic acid	50	—	—	—	—	+	DN U.V.-03
91	10	84	81	72	40	Anthranilic acid	33	09	—	57	++	+	D3-08, DB-41
79	02	69	42	11	02	*m*-Aminobenzoic acid	33	10	—	—	++	+	D3-08, DB-42
85	02	77	42	27	05	*p*-Aminobenzoic acid	—	08	—	58	++	+	
88	92	87	87	76	63	*p*-Aminoacetophenone	—	07	06	—	+	—	DN U.V.-33
91	04	90	43	38	06	2-Chloro-4-aminobenzoic acid	—	07	—	59	+	+	
87	18	89	93	83	82	*o*-Toluic acid	—	—	—	—	—	+	
92	05	85	74	78	28	6-Amino-*o*-toluic acid	38	10	—	—	+	+	D1-08, DB-27
85	25	83	91	78	79	*m*-Toluic acid	—	—	—	—	—	+	
90	15	93	86	81	64	2-Amino-*m*-toluic acid	38	11	—	17	++	+	D1-06, D2-07 D3-64, DB-38 Mo-71
87	34	78	89	83	80	*p*-Toluic acid	—	—	—	—	—	+	
92	91	96	96	93	95	*p*-Tolualdehyde	—	—	03	—	+	—	DB-32
93	54	89	88	89	73	3,5-Dinitro-*p*-toluic acid	65	—	—	58	—	++	Mo-30
88	38	88	92	89	89	*p-tert.*-Butylbenzoic acid	—	10	—	—	—	+	
93	91	95	94	92	93	Cumaldehyde	—	—	13	—	+	—	

Most of the compounds listed in this Table gave no reaction with D1, D2, D3, DB and Mo reagents; for exceptions see Remarks.

in order to demonstrate the effect that *ortho* and *para* substitution in benzoic acid has on the R_F values and on the colour reactions. The decrease or increase of the R_F value when certain substituents are introduced followed the same principle, as demonstrated for dihydric phenols (see above). For instance reference may be made to the great differences in R_F shown by compounds with nitro-, amino- and hydroxy-groups, in the case of solvents B, C and D. The differences in effect occurring in solvent E are not so marked except when an additional hydroxy-group is present.

In Table 23 (p. 72), five examples are also given of salicylic acid derivatives with different substituents at position 5. The variations in R_F values followed the general rule as described previously for dihydric phenols. Colour reactions in U.V. and with ferric chloride were observed throughout, but the shades were slightly different. Of

References p. 74. *(Continued on p. 70)*

TABLE 8

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SOME AROMATIC NON-PHENOLIC MONOCARBOXYLIC ACIDS WITH THE COOH GROUP IN THE SIDE CHAIN, AND THEIR DERIVATIVES

R_F values × 100						Compounds	Detection					Remarks
F	E	A	B	C	D		U.V.	D4	DN	Fe	Mn	Ind	
92	92	95	94	92	92	Phenylacetic acid	—	—	—	—	—	+	
81	14	85	63	40	03	*dl*-Mandelic acid	—	—	—	+	+	++	
25	02	03	03	04	00	Benzilic acid	34	06	—	—	+	++	Mo-71
95	27	95	96	89	88	*trans*-Cinnamic acid	—	—	—	03	++	+	
91	12	88	91	84	85	Hydrocinnamic acid	—	—	—	—	—	+	
87	84	92	93	90	87	Cinnamic alcohol	—	—	—	—	+	—	
88	86	91	93	89	87	Cinnamaldehyde	—	—	06	—	+	—	Mo-+
92	16	92	92	84	00	*o*-Nitrocinnamic acid	65	—	—	—	+	+	
94	13	89	88	90	64	*p*-Nitrocinnamic acid	65	—	—	—	+	+	
94	93	89	92	93	90	*p*-Nitrocinnamic acid ethyl ester	65	—	—	—	+	—	
92	03	84	62	32	07	*p*-Aminocinnamic acid	38	06	—	—	+	+	D1-08
87	32	89	81	67	25	*dl*-Phenyllactic acid	—	07	—	51	+	++	
88	29	92	54	68	25	Phenylpyruvic acid	—	06	06	42	+	+	D1-17, D2-06 D3-22
87	14	90	73	65	23	α-(3-Indolyl)-acetic acid	—	07	—	—	++	+	D3-03, DB-58 17
92	15	92	83	72	42	β-(3-Indolyl)-propionic acid	—	08	—	—	+	+	D1U.V.-33
94	17	93	88	83	50	γ-(3-Indolyl)-butyric acid	—	08	—	—	+	+	D1U.V.-33

Most of the compounds listed in this Table gave no reaction with D1, D2, D3, DB and Mo reagents; for exceptions see Remarks.

TABLE 9

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SOME AROMATIC NON-HYDROXYLATED DI-, TRI- AND TETRACARBOXYLIC ACIDS AND THEIR DERIVATIVES

R_F values × 100						Compounds	Detection					Remarks
F	E	A	B	C	D		U.V.	DN	Fe	Mn	Ind	
81	00	62	38	08	00	Phthalic acid	—	—	—	—	+	
46	05	13	15	00	00	Hexahydrophthalic acid	—	—	—	—	+	
88	93	87	95	88	87	Phthalaldehyde	33	03	—	—	—	
82	02	78	44	19	02	Homophthalic acid	33	—	—	—	+	D1-06, D2-17 D3-17, DB-17
82	00	70	32	09	00	Phthalonic acid	33	+	03	+	++	
87	03	87	50	26	00	4-Chlorophthalic acid	34	—	—	—	++	
90	00	90	90	85	68	3-Nitrophthalic acid 1-monoethyl ester	13	—	—	—	++	
80	02	73	36	12	00	4-Nitrophthalic acid	65	—	—	—	++	Mo-71
86	03	70	12	15	00	3,5-Dinitrophthalic acid	65	—	—	—	++	
83	00	53	00	00	00	3-Aminophthalic acid	38	—	—	—	—	D4-09
84	79	29	01	00	00	4-Aminophthalic acid	71	—	—	—	—	D4-06
89	00	87	50	00	00	Isophthalic acid	—	—	—	—	+	
91	00	89	03	02	00	Terephthalic acid	—	—	—	—	+	
69	00	22	02	00	00	Hemimellitic acid	—	—	—	—	+	
91	00	71	04	01	00	Trimesic acid	—	—	—	—	+	
58	00	18	00	00	00	Pyromellitic acid	—	—	—	—	+	

Most of the compounds listed in this Table gave no reaction with D1, D2, D3, D4, DB and Mo reagents; for exceptions see Remarks.

TABLE 10

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SOME AROMATIC AND HETEROCYCLIC AMINO ACIDS

R_F values × 100						Compounds	Detection										
F	E	A	B	C	D		U.V.	D1	D2	D3	D4	DB	NH*	Fe	Mo	Mn	Ind
28	00	00	00	00	00	L-Tyrosine	—	08	60	64	63	—	24	—	71	+	—
39	00	00	00	00	00	Monochloro-L-tyrosine	—	—	—	—	60	—	24	—	—	++	—
16	00	00	00	00	00	DL-*o*-Tyrosine	34	—	57	13	09	35	64	—	—	++	—
12	00	00	00	00	00	DL-*m*-Tyrosine	34	—	08	12	09	33	65	—	—	++	—
53	01	01	00	00	00	3,5-Diiodotyrosine	—	17	—	—	07, 23	—	24	—	—	++	—
26	04	00	00	00	00	DL-Phenylalanine	34	—	—	—	06	—	24	—	—	—	—
08	01	00	00	00	00	DL-3,4-Dihydroxyphenylalanine	—	06	06	06	61	28	24	42	03	++	—
06	00	00	00	00	00	DL-Proline	—	72	72	72	02	56	06	—	—	+	—
03	00	00	00	00	00	DL-4-Hydroxyproline	—	72	72	72	02	56	09	—	—	+	—
47	00	07	09	01	00	2,2′-Pyrrolidonecarboxylic acid	—	—	—	—	—	—	22	—	—	—	++
36	03	00	00	00	00	L-Tryptophan	—	—	—	—	06	—	24	—	—	+	—
72	11	62	86	48	42	Quinaldic acid	33	—	—	—	—	—	—	03	—	+	++
61	03	09	02	00	00	Kynurenic acid	40	—	—	—	—	—	—	—	—	—	+
35	01	08	28	04	02	Picolinic acid	—	—	—	—	—	—	+	—	—	—	+
67	00	31	56	05	01	Nicotinic acid	—	—	—	—	—	—	—	—	—	—	++
24	00	01	00	00	00	Chelidamic acid	—	—	—	—	—	—	—	03	—	—	++
62	00	09	00	00	00	Citrazinic acid	44	10	14	22	63	25	—	—	71	—	+

* NH = ninhydrin reagent.
DN reagent gave no reaction with the compounds listed in this Table.

TABLE 11

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SOME BIOLOGICALLY ACTIVE NITROGEN COMPOUNDS

R_F values × 100						Compounds	Detection										
F	E	A	B	C	D		U.V.	D1	D2	D3	D4	DB	DN	Fe	Mo	Mn	Ind
36	85	00	03	00	00	Ephedrine	—	—	—	60	08	—	—	—	—	+	—
28	73	00	00	00	00	Tyramine	33	12	57	10	06	—	—	—	—	++	—
23	00	00	00	00	00	Adrenaline	—	13	63	65	66	24	—	42	26	+	—
14	00	00	00	00	00	Noradrenaline	—	12	14	14	55	25, 69	—	50	28	+	—
92	90	94	93	94	92	Indole	—	06	—	—	10	25	15	—	—	+	—
53	84	01	09	00	01	Gramine	—	—	07	07	11	24	—	—	03	+	—
38	64	00	00	00	00	Serotonine	65	20	24	25	63	35	—	70	28	+	—
92	00	91	84	67	28	Isatin	65	07	08	60	—	—	03	—	—	+	—
24	16	00	01	00	00	Pyridoxine	32	09	24	25	12	35	—	64	71	++	—
16	53	05	31	00	00	Imidazole	—	—	08	12	07	+	—	59	03	+	—
47	00	08	02	01	00	Barbituric acid	34	03	13	15	07	+	—	62	—	++	++
33	02	02	00	00	00	Alloxantin	33	—	—	—	—	—	—	—	71	—	+
08	00	00	00	00	00	Uric acid	—	—	—	—	08	12	—	—	28	—	—
25	12	04	01	00	00	Uracil	—	—	—	—	—	—	—	—	—	+	—
25	14	02	08	00	00	Dehydrouracil	—	—	—	—	—	—	—	—	—	+	—

TABLE 12

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SOME COUMARIN DERIVATIVES

R_F values × 100						Compounds	Detection										
F	E	A	B	C	D		U.V.	D1	D2	D3	D4	DB	DN	Fe	Mo	Mn	Ind
96	87	84	91	87	89	Coumarin	—	—	—	—	—	—	—	—	03	++	—
93	84	92	97	94	92	3,3-Methylene-bis-4-hydroxycoumarin	—	—	—	—	—	—	—	—	—	+	—
87	62	84	85	73	18	Umbelliferone	44	07	17	64	64	43	—	—	—	+	—
88	66	93	92	76	26	3-Methyl-umbelliferone	44	01	13	65	10	71	—	—	—	+	—
87	63	88	87	72	18	4-Methyl-umbelliferone	33	09	15	21	—	56	—	—	—	+	—
86	62	88	87	73	17	4-Methyl-7-acetoxycoumarin	33	—	—	58	—	—	—	—	—	—	—
87	64	89	88	76	25	4-Methyl-6-acetyl-7-hydroxycoumarin	38	08	57	13	58	70	—	09	—	+	—
89	69	90	90	92	92	4-Methyl-7-hydroxy-8-acetylcoumarin	05	05	—	58	—	03	—	—	01	—	—
90	13	85	85	61	08	4-Hydroxycoumarin	34	06	19	21	08	—	—	—	—	+	—
33	01	03	01	00	00	Esculetin	38	—	—	—	61	51	—	—	—	+	—
81	23	77	53	12	00	4-Methylesculetin	01	02	06	07	64	51	—	45	01	+	—
69	66	55	35	45	39	4,5,7-Trihydroxycoumarin-6-carboxylic acid ethyl ester	32	09	64	65	07	69	—	61	30	+	—

TABLE 13

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SOME 1,2- AND 1,4-PYRONE DERIVATIVES

R_F values × 100						Compounds	Detection										
F	E	A	B	C	D		U.V.	D1	D2	D3	D4	DB	DN	Fe	Mo	Mn	Ind
84	13	97	97	93	93	Dehydroacetic acid	—	—	—	—	—	—	—	03	—	+	+
87	10	81	77	58	14	Isodehydroacetic acid	—	—	—	65	—	—	—	—	—	+	+
73	49	48	39	75	56	Dehydroacetic-5-carboxylic acid	—	—	63	64	+	—	—	63	71	+	+
77	03	87	83	23	01	Triacetic acid lactone	—	—	—	09	01	69	—	+	—	+	+
51	05	22	16	02	01	Kojic acid	40	08	22	25	11	68	—	65	42	+	—
83	00	78	70	46	13	Patulin	34	08	64	63	03	—	—	—	—	++	—
91	00	83	90	00	00	Coumalic acid	48	—	08	17	+	—	—	—	—	+	+
16	00	03	00	00	00	Chelidonic acid	—	—	—	—	+	—	—	—	—	+	++
95	96	92	95	91	93	Chelidonic acid diethylester	—	—	57	60	+	—	—	—	—	++	—
07	00	03	00	00	00	Meconic acid	34	58	—	24	17	—	—	64	—	++	++

TABLE 14

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SOME NATURALLY OCCURRING TROPOLONES

R_F values × 100						Compounds	Detection										
F	E	A	B	C	D		U.V.	D1	D2	D3	D4	DB	DN	Fe	Mo	Mn	Ind
96	93	94	97	93	93	α-Thujaplicin	65	11	—	64	15	—	52	51	—	+	+
97	93	93	97	93	94	β-Thujaplicin	65	12	—	65	65	—	—	62 / 49	—	++	++
92	92	90	97	95	89	γ-Thujaplicin	65	—	—	—	23	—	—	—	—	+	—
93	91	92	97	95	90	Nootkatin	65	—	—	—	—	—	—	—	—	+	—
94	32	94	96	92	92	Thujic acid	39	—	—	—	—	—	—	03	—	+	—
63	00	38	05	02	00	Puberulic acid	—	—	—	—	—	—	—	—	—	+	—
34	02	06	02	02	00	Puberulonic acid	15	58	—	—	08	—	07	53	03	+	—
84	00	50	07	03	00	Stipitatic acid	38	12	65	65	11	—	—	52	—	+	+

TABLE 15

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SOME NATURAL PRODUCTS OF VEGETABLE ORIGIN

R_F values × 100						Compounds	Detection										
F	E	A	B	C	D		U.V.	D1	D2	D3	D4	DB	DN	Fe	Mo	Mn	Ind
90	85	91	78	79	27	Pinosylvin	33	10	65	23	12	69	02	—	71	+	—
95	91	92	95	90	90	Pinosylvin monomethyl ether	—	08	65	23	12	38	02	—	—	++	—
90	87	95	92	90	77	α-Conidendrin	34	62	60	56	56	—	—	—	30	++	—
90	88	95	93	89	79	β-Conidendrin	39	62	62	56	60	34	—	—	34	+	—
89	88	91	96	87	78	Matairesinol	—	13	62	61	65	32	02	—	30	++	—
88	86	89	97	86	79	Pinoresinol	—	12	62	61	65	35	02	—	30	++	—
92	91	95	72	74	12	Nordihydroguaiaretic acid	—	14	23	25	64	29	55	51	30 57	++	+
74	51	42	00	00	00	Haematoxylin	65	07	28	29	12	25 64	17	66 25	54 28	67	—
89	68	93	87	71	46	Curcumin	10	—	64	64	59	54	—	64	62 23	++	—
68	01	13	00	00	00	Chlorogenic acid	34	03	56	07	07	42	—	42	32	+	+
56	02	09	00	00	00	Ellagic acid	70	—	57	57	56	56	—	71	69	+	+
95	93	92	29	40	00	Phloretin	13	09	23	65	12	66	—	23	28	++	—
73	23	54	00	00	00	Phlorizin	—	09	23	65	12	66	—	24	28	++	—
93	12	92	80	76	19	Hesperetin	—	03	13	64	08	28	—	—	—	+	—
01	03	00	00	00	00	Hesperidin	—	03	13	64	08	28	—	—	—	+	—
45	02	08	05	00	00	Hesperidin methyl chalcone	48	58	62	64	08	50	—	56	39	+	—
32	00	00	00	00	00	Rutin	65	06	65	64	11	48	—	50	07 69	+	—
92	14	91	05	04	00	Quercetin	48	06	63	64	11	48	—	50	03 43	+	—
94	21	90	05	09	00	Morin	10	60	65	66	11	03	—	51	51	++	—
90	00	38	00	00	00	m-Digallic acid	—	—	19	17	08	03	—	68	03	++	+
97	18	93	89	82	25	Cetraric acid	13	17 24	—	18	07	—	+	24	34	+	+
95	67	93	88	73	00	Gyrophoric acid	—	08	22	24	09	25	+	—	71	+	+

TABLE 16

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF MISCELLANEOUS COMPOUNDS

R_F values × 100						Compounds	Detection										
F	E	A	B	C	D		U.V.	D1	D2	D3	D4	DB	DN	Fe	Mo	Mn	Ind
93	04	91	91	86	51	3,5-Dihydroxy-2-carbethoxy-4-carboxyphenylacetic acid ethyl ester	33	07	19	23	10	25	—	17	71	—	+
94	85	93	94	92	92	3,5-Dihydroxy-2,4-dicarbethoxy-phenylacetic acid ethyl ester	33	07	15	22	09	25	—	—	71	—	—
93	02	96	17	24	00	Aurintricarboxylic acid	65	—	—	—	—	55	—	24	66 71	67	++

TABLE 17

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SOME METABOLIC PRODUCTS FROM MOULDS AND *Penicillium* SPECIES

R_F values × 100						Compounds	Detection										
F	*E*	*A*	*B*	*C*	*D*		*U.V.*	*D1*	*D2*	*D3*	*D4*	*DB*	*DN*	*Fe*	*Mo*	*Mn*	*Ind*
94	05	78	57	23	07	γ-Methyltetronic acid	—	01	10	12	08	21	—	64	—	+	+
87	23	70	40	35	03	α-Bromo-γ-methyltetronic acid	33	01	15	20	08	22	—	65	—	+	+
27	00	03	03	02	00	Carolinic acid	—	02	—	—	—	—	—	07	—	+	+
54	16	31	71	39	37	Carolic acid	—	—	—	—	—	—	03	07	—	+	+
08	00	03	04	00	00	Dehydrocarolic acid	—	—	—	—	—	—	—	07	—	+	+
33	00	08	17	02	00	Carlic acid	—	02	—	—	—	—	—	06	—	+	+
52	03	19	10	10	02	Carlosic acid	—	—	—	—	—	—	—	07	—	+	+
87	47	84	98	88	87	Terrestric acid	—	06	—	17	—	—	—	06	—	++	+
88	85	90	85	71	41	Penicillic acid	—	—	+	+	—	—	—	—	—	++	+
28	00	00	00	00	00	Ascorbic acid	—	—	05	05	—	—	+	72	30	+	+
25	01	00	00	00	00	Dehydroascorbic acid	71	—	—	—	12	—	—	—	—	+	—
68	69	42	52	06	01	Terrein	65	—	—	—	—	57	05	—	—	++	—
51	05	22	16	02	01	Kojic acid	40	08	22	25	11	68	—	65	42	+	—
81	49	67	80	68	61	Maltol	—	—	—	—	03	—	—	24	71	++	—
83	00	78	70	46	13	Patulin	34	08	64	63	03	—	—	—	—	++	—
79	00	77	38	65	45	Spinulosin	65	—	—	64	06	—	—	53	—	+	—
75	02	58	03	02	00	Gentisic alcohol	34	52	56	52	51	70	—	+	42 30	++	—
97	97	95	82	80	42	Gentisic aldehyde	33	—	—	—	60	70	07	28	30	+	—
88	36	82	19	25	00	Gentisic acid	38	—	—	—	60	70	—	28	68	+	+
88	62	78	90	85	68	6-Methylsalicylic acid	33	08	08	62	09	35	—	23	—	+	+
89	85	90	35	42	05	Toluhydroquinone	—	56	—	—	56	—	—	—	71	+	—
79	00	54	02	00	00	3,5-Dihydroxyphthalic acid	33	07	—	21	10	27	—	65	—	+	+
78	00	62	05	00	00	3,5-Dihydroxy-6-methoxyphthalic acid	33	07	—	19	09	25	—	25 65	01	++	+
94	90	91	38	43	05	Orcinol	34	07	15	65	12	26	—	—	68	+	—
93	28	93	47	52	02	Orsellinic acid	—	11	14	22	08	25	—	24	—	++	+
34	00	14	03	00	00	*cis*-Ethylene oxide dicarboxylic acid	—	—	—	—	—	—	—	—	—	24	+
83	00	60	08	05	00	3-Hydroxyphthalic acid	32	—	—	57	09	35	—	24	—	+	+
84	26	78	10	03	01	$C_{10}H_{10}O_6$-acid	34	—	—	21	10	27	—	65	—	++	+
88	46	85	28	18	00	$C_{10}H_{10}O_7$-acid	71	58	—	18	59	26 70	04	65	01	++	+
83	22	78	13	03	00	Ustic acid	33	—	—	21	10	26 52	—	25 65	33	++	+
85	36	79	26	11	00	Dehydroustic acid	65	17 58	—	17	10	—	—	25	—	++	+
80	04	82	90	25	03	Cyclopolic acid	—	—	—	17	+	—	05	—	—	+	+
91	02	89	93	91	57	Cyclopaldic acid	34	—	—	—	—	—	+	61	—	+	—
89	00	84	35	27	04	3,5,6-Trihydroxy-4-methyl-phthal-aldehyde	65	—	—	—	—	—	08	51	71	+	—
91	17	89	93	92	80	Mycophenolic acid	—	—	—	—	—	—	—	25	—	++	+
93	90	88	92	89	82	*d*-Usnic acid	24	—	—	—	08	—	—	58	—	—	+
93	20	92	93	79	23	Erdin	65	—	—	—	—	33	—	70	—	++	+
92	91	90	96	95	92	Geodin	65	69	—	—	—	—	—	—	01	++	+
86	85	81	93	89	84	Griseofulvin	39	07	—	17 24	—	—	—	71	26	++	—
63	00	38	05	02	00	Puberulic acid	—	—	—	—	—	—	—	—	—	+	+
34	02	06	02	02	00	Puberulonic acid	15	58	—	—	08	—	07	53	03	+	+
84	00	50	07	03	00	Stipitatic acid	38	12	65	65	11	—	—	52	—	+	+

TABLE 18

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SOME UNSATURATED ALIPHATIC MONO-, DI- AND TRICARBOXYLIC ACIDS

R_F values × 100						Compounds	Detection			Remarks
F	E	A	B	C	D		Fe	Mn	Ind	
90	06	87	84	73	35	Acrylic acid	—	++	++	
93	05	87	88	93	94	Crotonic acid	—	++	+	
89	13	90	88	84	83	Tiglic acid	—	++	+	
91	08	93	89	92	70	β,β′-Dimethylacrylic acid	—	++	+	
92	15	91	91	93	00	Sorbic acid	—	++	+	
70	00	46	18	02	00	Maleic acid	58	++	++	
85	00	68	16	00	00	Fumaric acid	60	++	++	
82	08	74	18	07	00	Acetylenedicarboxylic acid	—	++	++	
92	00	86	31	13	00	Mesaconic acid	—	++	++	
79	00	59	23	03	00	Itaconic acid	18	+	+	DB-70
71	01	46	25	03	00	Citraconic acid	57	++	++	
79	00	58	19	02	00	Glutaconic acid	18	++	++	
78	00	51	02	00	00	*trans*-Aconitic acid	—	++	++	D1-D4 +, Mo-71
85	00	44	01	00	00	*cis*-Aconitic acid	—	++	++	
90	00	75	23	00	00	Muconic acid	—	+	+	
69	00	22	01	00	00	Tricarballylic acid	—	—	++	

Most of the compounds listed in this Table gave no reaction with the following reagents: U.V., D1, D2, D3, D4, DB, DN and Mo; for exceptions see Remarks.

TABLE 19

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SOME ALIPHATIC DICARBOXYLIC ACIDS AND CORRESPONDING METHYL DERIVATIVES

R_F values × 100						Compounds	Detection	
F	E	A	B	C	D		Mn	Ind
66	00	30	04	00	00	Oxalic acid	+	++
69	03	32	08	01	00	Malonic acid	—	++
72	07	39	14	01	00	Succinic acid	—	+
78	07	57	34	03	00	Glutaric acid	—	+
79	08	67	55	04	01	Adipic acid	—	+
84	00	71	76	27	02	Pimelic acid	—	+
92	00	88	79	38	01	Suberic acid	—	+
88	00	88	79	68	02	Azelaic acid	—	+
76	00	50	12	03	00	Methylmalonic acid	—	++
81	00	68	28	13	00	Dimethylmalonic acid	—	++
82	00	67	22	05	00	α-Methylsuccinic acid	—	+
86	01	79	41	13	00	α-Dimethylsuccinic acid	—	+
74	02	70	12	00	00	Tetrafluorosuccinic acid	—	+
90	00	80	41	11	00	α-Methylglutaric acid	—	+
85	00	76	38	09	00	β-Methylglutaric acid	—	+
93	06	85	83	77	00	*d*-Camphoric acid	—	+
92	06	62	93	94	92	Pinonic acid	—	+
94	00	84	74	46	07	Pinic acid	—	+

The compounds listed in this Table gave no reaction with the following reagents: U.V., D1, D2, D3, D4, DB, DN, Fe and Mo.

TABLE 20

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SOME ALIPHATIC HYDROXY ACIDS

R_F values × 100						Compounds	Detection			Remarks
F	E	A	B	C	D		Fe	Mn	Ind	
51	01	14	06	00	00	Glycolic acid	—	+	++	
68	00	35	15	04	00	Lactic acid	—	+	++	
13	00	00	00	00	00	Hydracrylic acid	—	+	++	
35	00	03	00	00	00	Glyceric acid	—	++	++	
52	02	10	01	00	00	Tartronic acid	+	++	++	
48	00	07	02	00	00	DL-Malic acid	03	+	++	D3 +
32	00	02	00	00	00	DL-Tartaric acid	44	+	++	
47	00	06	00	00	00	Dihydroxytartaric acid	58	+	++	
48	03	05	00	00	00	Citric acid	03	+	++	
40	00	03	00	00	00	Isocitric acid	—	—	+	
05	00	00	00	00	00	Mucic acid	—	+	+	
05	00	00	00	00	00	D-Galacturonic acid	—	+	+	
07	00	02	00	00	00	D-Glucuronic acid	—	+	+	
27	00	01	01	00	00	D-Glucuronic acid lactone	—	+	—	
05	00	00	00	00	00	D-Gluconic acid	03	+	++	D1-7, D2-17, Mo-30
28	00	01	00	00	00	D-Gluconic acid-δ-lactone	—	+	—	
71	03	32	19	01	00	2-Oxogulonic acid	—	+	+	

Most of the compounds listed in this Table gave no reaction with the following reagents: U.V., D1, D2, D3, D4, DB, DN and Mo; for exceptions see Remarks.

TABLE 21

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SOME ALIPHATIC KETO ACIDS AND OTHER KETO AND HYDROXY COMPOUNDS

R_F values × 100						Compounds	Detection									
F	E	A	B	C	D		U.V.	D1	D2	D3	D4	DB	DN	Fe	Mo	Mn	Ind
92	35	87	93	77	46	Pyruvic acid	—	08	62	24 64	09	—	03	17	71	+	++
83	00	64	00	00	00	α-Ketobutyric acid	—	—	07	65 60	02	—	03	71	71	++	++
65	00	31	08	00	00	α-Ketoglutaric acid	—	—	07 62	65	01	—	03	60	—	+	++
79	01	44	04	00	00	β-Ketoglutaric acid	—	07	65	56	60	—	+	63	71	++	++
76	00	45	13	01	00	γ-Ketopimelic acid	—	—	—	—	—	—	+	—	—	—	++
78	04	62	71	29	09	Laevulinic acid	—	—	+	+	—	—	03	—	—	—	+
23	00	00	00	00	00	Mesoxalic acid	34	—	—	—	—	—	+	03	—	+	++
42	03	03	02	00	00	Oxalacetic acid	34	07	24 64	15 25	09	—	+	60	71	+	++
97	85	95	95	90	75	Ketipic acid monoethyl ester	34	—	—	62	62	—	+	13	71	—	+
94	88	93	94	92	90	Ketipic acid diethyl ester	33	17	68	55	08	—	+	64	71	++	++
92	93	96	92	93	95	Parasorbic acid	34	—	13	21	—	—	—	—	—	+	—
48	00	11	01	00	00	*trans*-Aconitic acid anhydride	47	—	52	20	07	—	—	—	—	++	++
Miscellaneous keto and hydroxy compounds																	
95	29	33	28	01	00	1,3-Dihydroxyacetone	—	—	—	—	—	—	05	—	71	+	—
85	82	82	93	80	66	Diacetyl	—	06	64	64	—	—	06	—	30	+	—
85	08	91	84	66	20	Acetoin	—	03	+	—	—	18	06	—	—	+	—
77	70	49	73	23	06	Acetaldol	—	—	66	65	—	—	06	—	—	+	—

these compounds, 5-aminosalicylic acid was the most reactive, whereas 5-hydroxysalicylic acid showed a typical hydroquinone colour pattern.

In Table 24 (p. 72), three typical colour-reaction sequences are presented, which may serve as a guide when determining, with colour reactions only, whether a substanec is a pyrocatechol, resorcinol or hydroquinone derivative. As far as the examples from the reference system are concerned, these colour patterns were fairly constant and were not influenced by additional substituents. An additional hydroxy group forms an exception, since it gives colour patterns identical to those of trihydric phenols.

Naphthols show intense colour patterns of the type indicated. Their reactions with DB and Fe are also strong, although the colour varies; this has, however, not been indicated in the Table. It was striking how easily the aliphatic keto acids reacted with diazonium reagents. However, the colours were more or less equal in shade, and it was difficult to discern any distinct differences, except with ferric chloride.

Four stabilized diazonium reagents were chosen from commercial sources. During the testing of several diazonium reagents (some of which are described in the section *Additional reagents*) it was observed that many of them failed to react under standard conditions, the principle of which is that diazo coupling occurs when the *para* or *ortho* position in a phenolic structure is free. Other reagents gave fairly specific colours with certain types of compounds. As can be seen from the Tables, the choice of at least 4 different diazonium reagents is justified, since in many cases only one reagent gives a positive reaction. Phenols substituted in the *meta* position are in all cases favoured. Coupling occurred even if the hydroxyl group was etherified. Pyrones, pyridoxine, barbituric acid and aliphatic keto acids are some of the non-phenolic compounds that undergo diazo-coupling.

2,6-Dibromoquinone-4-chloroimide is known to be a typical phenolic reagent, coupling predominantly with phenols with a free *para* position. With a few exceptions, reaction also occurred when the group in the *para* position could be decarboxylated or oxidized into a volatile aldehyde[18]. Some examples of other types of coupling compounds are *o*- and *m*-aminobenzoic and -toluic acids, indole, pyridoxine, and kojic acid. Very rarely a red colour was produced, for instance with γ-methyltetronic acid, thiosalicylic acid and uric acid.

2,4-Dinitrophenylhydrazine was used for detecting aldehyde and keto compounds. Some examples of other compounds giving positive reactions are haematoxylin, α-thujaplicin, indole, pyrogallol and pyrocatechol.

The colour reactions of ferric chloride with phenolic compounds are usually described as rather unspecific. However, most pyrocatechol derivatives gave green colours. Many *meta*-substituted phenols of resorcinol type failed to give colours. This was even the case with simple monohydric phenols, probably due to the low concentration level used. Some examples of non-aromatic compounds with a positive reaction are tetronic acids and some aliphatic acids, which gave yellow colours.

Phosphomolybdic acid has been shown to give many colour shades, which were difficult to interpret.

Most compounds gave positive reactions with permanganate. Fully methylated

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phenols and methyl- and chloro-derivatives of benzoic acid, however, gave a negative reaction. The indicator reagent was specific for detecting carboxyl groups. The colour in U.V. of a non-sprayed compound was recorded. Since it was nonspecific, it was hard to interpret. Examination of the sprayed areas in U.V. yielded further data, but these have not been recorded in this paper.

TABLE 22

A COMPREHENSIVE LIST OF TYPES OF COMPOUNDS INVESTIGATED, WITH THEIR APPROXIMATE R_F VALUES (± 10) IN SIX DIFFERENT SOLVENT SYSTEMS

Approx. range of R_F values × 100						*Types of compounds*
F	*E*	*A*	*B*	*C*	*D*	
						Monohydric phenols
90	90	90	90	90	90	+ alkyl or OCH_3 or Cl
50	80	50	30	10	00	+ NH_2
90	70	90	90	90	70	+ 1-2 NO_2
90	70	90	30	50	70	+ 3 NO_2
90	90	90	70	70	40	+ CH_2OH
90	80	90	90	80	60	+ $COCH_3$
90	90	90	90	90	60	+ CHO
90	10	90	40	30	00	+ COOH
90	90	90	90	90	70	+ COOR
40	20	10	00	00	00	+ COOH + NH_2
80	10	60	20	30	00	+ COOH + NO_2
90	60	90	90	90	70	+ COOH + CH_3
						Dihydric phenols
90	90	90	60	60	10	1,2
90	90	90	30	30	00	1,3
90	90	90	20	20	00	1,4
80	10–30	80	10–30	10–30	00	1,2 + COOH
80	10–70	70	10	10–20	00	1,3 + COOH
80	40	80	20	20	00	1,4 + COOH
80	00	60	00	00	00	1,2 + 2 COOH
80	00	50	00	00	00	1,3 + 2 COOH
60	00	20	00	00	00	1,4 + 2 COOH
80	00–60	60	00	00	00	*Trihydric phenols*
70	00	40–80	00	00	00	+ COOH
90	90	90	50	50–70	10	*Dihydric naphthols*
						Benzoic acid derivs.
90	00	80	50–70	20–60	10–30	+ NH_2
90	25	80	90	90	80	+ CH_3
90	20–40	90	90	90	40–80	+ NO_2
80	00	30–80	10–40	00	00	+ 2-3 COOH
20–60	10	10–50	00–50	10	00	*Aromatic and heterocyclic amino acids*
20–50	10–80	10	00	00	00	*Biogenic amines*
90	20–80	80	80	70	30	*Coumarins*
80	10–50	10–90	10–90	10–80	10–80	*Pyrones*
90	90	90	90	90	90	*Tropolones*
40–80	10–40	10–40	10	00	00	+ COOH
20–90	10–40	10–70	10–70	10–40	10–40	*Tetronic acids*
						Aliphatic carboxylic acids
90	10	90	80	80	40–90	Monocarboxylic acids, unsaturated
80	00	60	20	10	00	Dicarboxylic acids, unsaturated
70–80	10	40–80	10–70	10–60	00	Dicarboxylic acids, saturated
10–60	00	10–30	10	00	00	Hydroxy acids
30–80	10–30	10–90	10–80	10–70	00–40	Keto acids

TABLE 23

THE INFLUENCE OF SUBSTITUENTS ON R_F VALUES AND COLOUR REACTIONS

1. A few examples are given of compounds 5-X-salicylic acid, where X designates the chloro-, methyl-, hydroxy-, carboxyl- and amino-group.

2. A few examples are given of compounds *ortho*-Y-benzoic acid, where Y designates the chloro-, methyl-, hydroxy-, amino-, thio-, nitro- and carboxyl-group.

3. A few examples are given of compounds *para*-Z-benzoic acid, where Z designates the chloro-, nitro-, methyl-, amino-, hydroxy- and carboxyl-group.

The influence of these substituents on the R_F values as well as on the characteristics of the colour reactions is clearly demonstrated.

R_F values × 100						Compounds	Detection										
F	*E*	*A*	*B*	*C*	*D*		*U.V.*	*D1*	*D2*	*D3*	*D4*	*DB*	*DN*	*Fe*	*Mo*	*Mn*	*Ind*
94	67	86	87	85	70	5-Chlorosalicylic acid	33	—	—	—	08	—	—	25	—	+	+
91	46	89	90	89	65	5-Methylsalicylic acid	38	—	—	—	—	—	—	25	—	+	+
88	36	82	19	25	00	5-Hydroxysalicylic acid	38	72	—	—	58	70	—	28	68	+	+
86	02	92	24	13	00	5-Carboxysalicylic acid	30	—	—	—	—	—	—	65	—	—	+
41	21	09	00	00	00	5-Aminosalicylic acid	48	—	62	64	08	53	—	65	68	++	+
88	28	85	88	86	80	*o*-Chlorobenzoic acid	—	—	—	—	—	—	—	—	—	—	++
87	18	89	93	83	82	*o*-Methylbenzoic acid	—	—	—	—	—	—	—	—	—	—	+
92	51	92	87	86	52	*o*-Hydroxybenzoic acid	33	06	—	—	07	—	—	24	—	+	+
91	10	84	81	72	40	*o*-Aminobenzoic acid	33	—	—	08	09	41	—	57	—	++	+
92	11	92	78	88	33	*o*-Thiobenzoic acid	33	01	07	07	08	21	—	58	30	++	+
96	16	88	90	80	29	*o*-Nitrobenzoic acid	70	—	—	—	—	—	—	—	—	—	+
81	00	62	38	08	00	*o*-Carboxybenzoic acid	—	—	—	—	—	—	—	—	—	—	+
94	37	91	92	89	92	*p*-Chlorobenzoic acid	—	—	—	—	—	—	—	—	—	—	+
96	22	92	88	88	88	*p*-Nitrobenzoic acid	70	—	—	—	—	—	—	—	—	—	++
87	34	78	89	83	80	*p*-Methylbenzoic acid	—	—	—	—	—	—	—	—	—	—	+
85	02	77	42	27	05	*p*-Aminobenzoic acid	—	—	—	—	08	—	—	58	—	++	+
88	08	91	37	28	01	*p*-Hydroxybenzoic acid	—	06	—	58	07	—	—	03	—	+	+
91	00	89	03	02	00	*p*-Carboxybenzoic acid	—	—	—	—	—	—	—	—	—	—	+

TABLE 24

A LIST OF SOME TYPICAL COLOUR-REACTION SEQUENCES THAT ARE INDEPENDENT OF FURTHER SUBSTITUENTS

Few exceptions have so far been observed. (The code numbers have been given with a good approximation.)

Type of compounds	Detection										
	U.V.	*D1*	*D2*	*D3*	*D4*	*DB*	*DN*	*Fe*	*Mo*	*Mn*	*Ind*
Pyrocatechol derivatives		07	10	21	10	35		42		+	
		07	—	—	10	35		42		+	
Resorcinol derivatives		06	15	21	10	65		—		+	
		06	15	21	10	25		25		+	
Hydroquinone derivatives		72	—	—	72	53		—		+	
		72	72	72	72	69		28		+	
Naphthol derivatives		12	25	25	12	+		+		+	
Coumarin derivatives	33	+	+	+	+	51				+	
	44	+	+	+	+	71				+	
Benzoic acid, chloro- and methyl-derivatives only	—	—	—	—	—	—	—	—	—	—	+
Tetronic acids		—	—	—	—			07		+	+
Aliphatic keto acids		07	62	64	09	—	03	60	71	+	+

On comparing the data for an unknown compound with the data of the R_F values presented as a diagram in the reference system in the order F, E, A, B, C and D, it was possible visually to establish its probable type and to exclude several other types as not probable. The identity of six R_F values with those of an unknown compound was not regarded as significant, unless several colour reactions were found to agree. This provided strong evidence for the identity of a certain type of compound, even before it had been isolated. This information facilitates, in some cases, the chemical isolation procedure or the choice of the preparative chromatographic method.

In view of these possibilities, the reference system was provided with several cross-indexing systems.

The inclusion of many natural products of vegetable origin indicates further possibilities of investigation in this field.

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II. SUMMARY

A paper-chromatographic procedure is described for the identification of phenol derivatives, metabolic products from moulds, especially from *Penicillia* species, and aliphatic compounds of biochemical interest. Information is given concerning the application of the method to the characterization of unknown substances encountered in various biological investigations, mg quantities of material being sufficient. The R_F values in six different solvent systems together with the corresponding colour reactions produced by ten standard reagents have been collected and presented in 21 Tables. In all, approx. 450 compounds were investigated.

Original recordings were made on standard cards, which made it possible to present the R_F values as diagrams and to give the code of colour reactions for each compound separately. A standard numerical colour index was used for these colour recordings.

The possible application of this procedure, within the biochemical, microbiological, medical and organic-chemical fields, to preliminary investigations of unknown mixtures is indicated.

A special set-up was constructed for rapid analysis by means of which spraying as well as evaluation of six chromatograms could be carried out simultaneously.

Two new stable diazonium reagents, *o*-dianisidine diazotate and 4-benzoylamino-2,5-dimethoxy-aniline, were found to be useful for detecting phenolic compounds and aliphatic keto acids. Several other reagents were tested with regard to their usefulness for spraying purposes in paper-chromatographic analysis.

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METHODS FOR THE SEPARATION OF SOUTH AMERICAN STRYCHNOS AND INDIAN CURARE ALKALOIDS

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I. INTRODUCTION

In the chemical study of the active constituents of curares the research workers[13, 19, 21, 24, 26, 39, 51, 56, 58, 86, 87] are faced with a number of problems, which in spite of century-long investigation are not yet close to a definite solution.

These substances are alkaloids and are found in curares employed by numerous Amazonian tribes as arrow poisons, as well as in the bark of various species of *Strychnos*.

The first great difficulty lies in the fact that for many years the conventional methods did not allow an adequate separation, for analytical or preparatory purposes, of the many alkaloids to be found in the bark and roots of South American *Strychnos* species and in the curares derived therefrom.

The claim is well justified that also in this field the introduction of chromatographic methods has marked a turning point in the research.

In fact, it is due to HEINRICH WIELAND, who employed chromatography on an alumina column, that in 1941 the first pure alkaloids were obtained from calabash curares as well as from *S. toxifera*[91].

In 1952, SCHMID, KARRER and coworkers[85] introduced partition chromatography on a cellulose column for preparatory purposes. For analytical purposes they developed two-dimensional chromatography on paper using known alkaloids; by determining the rates of movement and colour reactions they supplied the basis for the identification of these substances. These methods have been adopted, with slight variations, by THEODOR WIELAND[94] and by MARINI-BETTÒLO and coworkers[1].

The difficulties inherent in the resolution of alkaloid mixtures by chromatographic methods only, whether on a column or on paper using one and two-dimensional systems, have subsequently led us to introduce the use of aqueous solvents in these methods[27], as well as electrophoretic techniques[59, 60], electrophoresis on cellulose columns and counter-current distribution[26].

The object of this review is to set forth and comment on the techniques and practical aspects of the various methods that make possible the solution of problems for which chromatographic methods alone appear to be insufficient.

An outline will, therefore, be given of:

1. Data concerning the starting material.
2. Preliminary assays for orientation purposes.
3. Methods for extracting all the alkaloids from curares and *Strychnos* species.

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4. Analytical separation of alkaloids by:
   (a) paper strip chromatography;
   (b) circular paper chromatography;
   (c) paper electrophoresis;
   (d) two-dimensional chromatography.

5. Preparative separation of alkaloids by:
   (a) absorption chromatography on alumina columns;
   (b) partition chromatography on cellulose columns;
   (c) separation on paper sheets;
   (d) band electrophoresis;
   (e) continuous electrophoresis;
   (f) column electrophoresis;
   (g) counter-current distribution.

2. STARTING MATERIAL

The following two types of material are usually employed for the extraction of alkaloids:

1. Curares prepared by Amazonian Indians.
2. Plants of the genus *Strychnos* from South America.

In both cases there may be considerable differences between the various laboratory materials available.

The curares which contain quaternary curarizing alkaloids of the indole group, are differentiated into: calabash curares and "pot curares"*. As commonly known, they are prepared by Amazonian and Orinoco Indians by boiling extracts from various plants, including *Strychnos* bark; a sticky black mass is thus obtained containing concentrated alkaloids together with other substances.

From data reported in the literature it may be assumed that calabash curares contain about 8–10% of quaternary curarizing alkaloids.

Work on curares, which undoubtedly are a valuable material owing to their high concentration of active principles, is somewhat hampered by the presence of other alkaloids from other plants added during the preparation.

A more interesting material may be obtained from the bark and roots of *Strychnos* species. This has not undergone other transformations and is substantially made up of most of the alkaloids to be found in Indian curares. The alkaloid percentage, however, is very low; on an average it lies between 0.1 and 0.2%, and exceptionally 1%.

On the other hand two main difficulties are likely to be met with when using botanical material: first the exact botanical determination of the plant; second the finding of a sufficient quantity of material of the same species, since one rarely finds

* One cannot at the present moment consider calabash curares as the only source of indole curarizing alkaloids. These alkaloids may be found in curares stored in various containers and even in glass bottles (see Symposium on Curare and Curare-like Agents, Rio de Janeiro, August 1957, Elsevier, Amsterdam, 1959).

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plants of the same species in one and the same region. This accounts for the fact that more than 1–2 kg of bark of a single species is not often available.

Furthermore, it must be borne in mind that for alkaloid extraction the part of the *Strychnos* bark close to the ground and the roots are useful, whereas neither the branches nor the fruit can be utilized, since according to our observations they do not contain alkaloids even when these are plentiful in the bark and roots.

Another difficulty is the possible variation, as was found in some cases, in the alkaloid compositon in one and the same species according to its geographical distribution and the season when it was gathered.

3. PRELIMINARY ASSAYS

Since the available material is so heterogeneous, it may be necessary—particularly for the plant collector—to have a method of detecting the presence of curarizing alkaloids.

This can be carried out, in a preliminary way, by treating a few hundred milligrams of material on a spot plate with 4–5 drops of 5% acetic acid (or 10% tartaric acid) and then absorbing the coloured liquid with a bit of filter paper within a few minutes[23]. As soon as the paper has dried it should be treated with a 1% ceric sulphate solution[46]. If indole curarizing alkaloids are present the paper will turn red or violet or blue or green.

This test is so simple and rapid that it can be carried out in the field much to the advantage of the material collector. He is thus spared the trouble of gathering plants and carrying them hundreds of km before finding that they are of no use from the chemical point of view.

This method is applied today in Brazil when gathering botanical material.

4. METHODS OF EXTRACTING ALKALOIDS

The process for the extraction of alkaloids is more or less the same whether the curares are prepared by Indians or obtained from plant bark, since in both cases the curarizing alkaloids have to be separated from inert substances and from tertiary alkaloids.

WIELAND utilized extraction with methanol followed by boiling water. The alkaloids were then precipitated with mercuric chloride and converted to the reinecke salts which were subjected to chromatography on alumina columns[89–91].

SCHLITTLER AND HOHL[80] in their work on *S. melinoniana* percolated the powdered bark first with water and afterwards with methanol containing 3% acetic acid. KING, on the other hand, usually employed a 3% aqueous solution of tartaric acid[48], while SCHMID AND KARRER[85] used methanol and acetic acid.

One or two percent acetic acid is more convenient than tartaric acid, because the latter has to be removed as alkaline tartrates, which is not necessary in the case of the former.

The extracts obtained by the above methods are concentrated to small volumes, brought to pH 8 with ammonia or alkali and extracted with chloroform to separate

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tertiary bases. The solution is then treated with dilute hydrochloric acid and the alkaloids are precipitated either as reineckates or picrates.

The reinecke salts of the quaternary and some of the tertiary bases can be fractionated owing to their different solubilities in acetone–water[85], though this method is not always reliable.

The reinecke salts can also be purified directly on alumina columns (WIELAND and SCHLITTLER), but today it is considered better to transform them into chlorides by the Kapfhammer method, *i.e.*, by treating them with silver sulphate and then with barium chloride.

BOEKELHEIDE[20] extracts the calabash curares with water and then with methanol. The concentrated liquids are brought to pH 8 and then extracted with methylene chloride, thus eliminating the tertiary bases. The quaternary bases are afterwards precipitated with picric acid.

The mixture of alkaloid picrates is passed over an ion exchange resin (Dowex-II-X4 Cl) to recover the quaternary alkaloid chlorides.

5. ANALYTICAL SEPARATION OF ALKALOIDS

(a) *Paper strip chromatography*

It must be pointed out here that the peculiarities of quaternary ammonium derivatives make it rather difficult to decide which solvents are best suited for these separations.

TABLE I

COMPOSITION OF SOLVENTS USED IN CHROMATOGRAPHIC SEPARATIONS OF *Strychnos* AND CURARE ALKALOIDS*

Solvent composition	*Designation*	*Author*	*Reference*
Acetic ester–water–pyridine 200:200:90	A	KARRER	82
Methyl ethyl ketone–water–cellosolve 300:70:15	B	KARRER	82
Methyl ethyl ketone saturated with water and 1–3% methanol	C	KARRER	85
	2	MARINI-BETTÒLO	27
Acetic ester–pyridine–water 7.5:2.3:1.65	D	KARRER	85
	1	MARINI-BETTÒLO	27
Acetic ester–acetic acid–water 7.5:0.9:0.9	E	KARRER	85
n-Butanol–chloroform–water 10:10:0.6	F	KARRER	85
Ethyl acetate–methyl ethyl ketone–glycol monomethyl ether–water 200:300:154:200	A	TH. WIELAND	92
Acetic ester–pyridine–water 200:90:200	B	TH. WIELAND	92
Ethyl acetate–pyridine–water 1000:600:300	C	TH. WIELAND	92
Ethyl formate–acetone–water 1000:1450:400	D	TH. WIELAND	92
Methyl ethyl ketone–pyridine–water 1400:300:300	E	TH. WIELAND	92
Methyl ethyl ketone saturated with water containing 10% methanol	3	MARINI-BETTÒLO	27
5 g of $CH_3COONa \cdot 3H_2O$ in 100 ml 10% acetic acid; the solution is saturated with amyl alcohol	4	MARINI-BETTÒLO	27
10 g $CH_3COONa \cdot 3H_2O$ in 100 ml 10% acetic acid; the solution is treated as above	5	MARINI-BETTÒLO	27
n-Butanol saturated with water		BOEKELHEIDE	20

* It is important to avoid traces of peroxides in solvents. For this reason methyl ethyl ketone must be distilled before use and then passed over an ion-exchange column (Dowex-50-X4) with ferrous ion as the cation.

Butanol must also be purified by washing it with sodium bisulphite.

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TABLE 2

R_C VALUES AND PHYSICO-CHEMICAL PROPERTIES OF ALKALOIDS FROM SOUTH AMERICAN *Strychnos* SPECIES AND CALABASH CURARES*

*Alkaloid***	R_C		*Colour*	*Fluorescence*	*Colour with $Ce(SO_4)_2$ (Spot plate)*	*Reference*
	C	*D*				
Erythrocurarine	0.12	0.31	Red	Red		64
Alkaloid A	0.23	0.55			Blue-violet	85
Solimoesine 3	0.27	—			Purple	63
Guiacurarine I	0.31	0.42	Brown-yellow		Blue-violet	64
Alkaloid B	0.34	0.51			Red-violet	85
Alkaloid C	0.34	0.51			Red-violet	85
Alkaloid D	0.35	0.68			Red-violet	85
Alkaloid E	0.36	0.58			Blue	85
Toxiferine I	0.42	0.67			Red-violet	85
Alkaloid F	0.49	0.73			Blue-violet	85
Guiacurarine 10	0.50	1.05			Violet	76
Alkaloid S chloromethylate	0.51	1.35			Purple-red	73
Macrophylline A	0.52	1.6			Violet	38
Rubrocurarine 4	0.52	0.50	Red	Red		64
Guiacurarine 8	0.58	0.56	Yellow	Yellow	Blue-violet	63
Rubrocurarine 3	0.63	0.54	Red	Red	Pale blue	64
Rubrocurarine 2	0.65	0.51	Red	Red		64
Alkaloid G	0.65	0.73			Blue	85
Fluorosolimoesine 2	0.67	1.2	Yellow	Yellow		63
Alkaloid R	0.68	0.92			Purple	73
Caracurine I	0.70				Purple-red	4
Alkaloid H	0.71	0.99			Red-violet	85
Erythrocurarine 3	0.72	1.00	Red	Red	Blue	76
Guiacurarine 9	0.73	0.94	Orange-red	Orange	Violet-blue	76
Guiacurarine 2	0.76	0.99			Violet-blue	64
Rubrocurarine 1	0.78	0.92	Red	Red		64
Fluorosolimoesine 3	0.79	3.1		Green-yellow		63
Fluorosolimoesine 4	0.79	2.0		Green-yellow		63
Caracurine II	0.8				Purple	4
Caracurine III	0.8	1.32			Purple-red	4
Calebassine (C-Toxiferine II = C-Strychnotoxine)	0.8	1.03			Blue-violet	85
Solimoesine 2	0.85	—			Red	63
Alkaloid I	0.89	1.06			Blue-violet	85
Solimocurarine	0.93	0.82			Blue-violet	63
Curarine	1.00	1.00			Blue	85
"Rotes Alkaloid"			Red			85
Caracurine IV	1.00				Violet	4
Guiacurine I	1.05	1.34			Blue-violet	64
Guiacurarine 2	1.07	1.33			Blue	64
Caracurine Va	1.1				Purple-blue	4
Alkaloid J	1.04	1.12			Red-violet	85

(Contd. on p. 80)

* At present there is some confusion in the nomenclature of alkaloids from *Strychnos* bark and calabash curares.

To avoid these difficulties a Commission to unify the nomenclature of these alkaloids was appointed at the International Symposium on Curare and Curarizing Substances in Rio de Janeiro, August 1957.

In this review we have used the names given in the original papers, with the exception that WIELAND'S *C-Alkaloid* term has been simplified to *alkaloid*. The former term is now inaccurate because many C-alkaloids, *i.e.* calabash alkaloids, have been found in *Strychnos* species. Moreover the calabash alkaloids have also been found in other curares. The term C-alkaloid followed by letters was necessary in the past to avoid confusion with alkaloids from other plants. In this review no confusion is possible since only alkaloids of *Strychnos* and curare are referred to.

** The alkaloids are in the form of the chlorides unless otherwise stated.

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TABLE 2 (*Continued*)

Alkaloid	R_C C	R_C D	Colour	Fluorescence	Colour with $Ce(SO_4)_2$ (Spot plate)	Reference
Guaianine	1.12				Pale blue-violet	36
Caracurine IX chloromethylate	1.12				Violet	6
Alkaloid 2 fluorescent	1.18	1.05		Blue	Blue-violet	76
Alkaloid K (Dihydrotoxiferine)	1.22	1.14			Violet	85
Solimoesine I	1.3	1.54			Purple-violet	63
Caracurine V	1.4	—			Purple-red	4
Caracurine VII chloromethylate	1.40	—			Purple-orange	4
Erythrocurarine 2	1.43	1.15	Red	Red	No colour	64
Alkaloid M	1.45					3
Melinonine H	1.47					8
Melinonine K	1.52					8
Alkaloid Y	1.59	2.22			Red-violet	3
Caracurine VI	1.6				Purple	3
Macrophylline B	1.64	1.86			Violet	38
Calebassinine	1.68	1.38			No colour	85
Guiacurarine 3	1.70	1.64	Orange	Green-yellow	Green-blue	64
Diaboline	1.76	2.10			No colour	38
Fedamazine	1.9			Orange	Blue	7
Melinonine J	2.01					8
Melinonine F	2.01					8
Pseudofluorocurine	2.10		Yellow	Green-yellow	Orange-red	73
Caracurine VII	2.1				Orange	3
Fluorocurine	2.10	1.70	Yellow	Green-yellow	Orange	84
Fluorocordatine	2.13	1.20		Blue	Blue	76
Melinonine E	2.18					8
Fluorocurinine	2.23	1.65	Yellow	Yellow-green	Pale red	85
Fluorocurarine (C-Curarine III)	2.25	1.71	Pale yellow	Blue	Pale blue	85
Alkaloid UFC						85
Melinonine F	2.01					8
Melinonine M	2.42				Pale blue	8
Alkaloid L	2.50	1.99			Red	84
Desacetyldiaboline	2.64	2.42			Pink-red	76
Mavacurine	2.70	2.23			Red-violet	8,63
Precurarine	2.78	3.8			Blue	63
Fluorescent Alkaloid 1	2.90	1.69		Green-yellow	Pale red	76
Kryptocurine	2.95	3.10				72
Melinonine G	3.00					8
Fluorosolimoesine 1	3.00	1.5	Yellow	Yellow-green		63
Alkaloid X	3.5	4.9			Red	85
Alkaloid O	3.95	4.30			No colour	36
Premavacurine	4.0	3.6			Red-violet	63
Alkaloid P	—	—			Blue	36
Alkaloid 1						92
Alkaloid 2					Red-violet	92
Xanthocurine	—	1.58	Orange		Green-blue	36
Alkaloid UB		1.13			Brown	46
Alkaloid Q		4.92				73
Croceocurine			Orange-red			72
C-Strychnotoxine Ia	*					92
Nor-C-dihydrotoxiferine					Purple	6

* R_F 0.25 in TH. WIELAND'S solvent A.

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Various solvents have been tried out by different authors (see Table 1), but except special cases the most suitable are KARRER AND SCHMID'S C and D, as they give fair resolutions and most of the displacement values recorded in the literature have been obtained with these solvents.

If a two-dimensional system is used, these solvents give in many cases a fairly satisfactory separation of many alkaloids.

In Table 2 the R_C values (displacements calculated with respect to curarine) of these substances are given in both solvents C and D, as well as other characteristics by which they can be recognized. In some cases when displacement rates refer to other

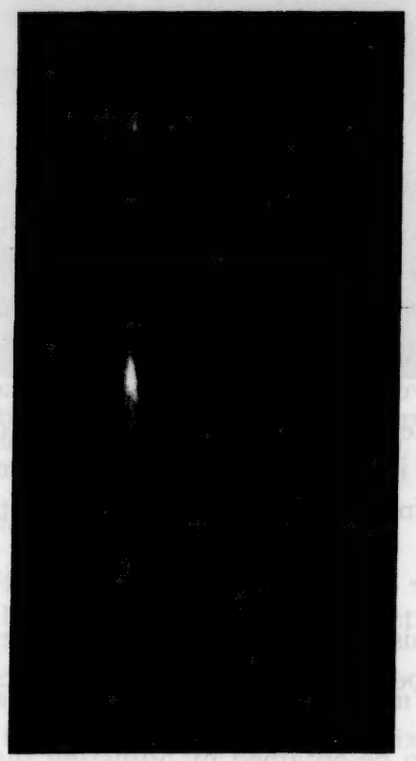

Fig. 1. Chromatograms of *S. parvifolia* alkaloids. Influence of the different weights of the sample on the R_F values.

alkaloids such as mavacurine or melinonine B, it is convenient to convert them into R_C values, by calculation, in order to attain a uniform expression.

In view of the low velocity of these alkaloids, chromatography must always be carried out continuously for 12 to 18 hours if good resolutions are to be attained.

The determination of R_C in the case of these alkaloids very often gives rise to considerable errors: it has been noticed, for instance, that although the same chromatographic conditions are maintained an increase in the quantity of the substances employed is sufficient to produce a strong variation in the R_C (Fig. 1).

SCHMID AND KARRER[85] had already pointed out that the R_C values are reproducible only when a 50 γ sample of alkaloid chloride is taken. Moreover, it is necessary that the standard curarine chloride should have a medium displacement of 11 cm for all

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alkaloids in solvent "C", and in solvent "D", 24 cm for alkaloids with $R_C < 1$, and 18 cm for those with $R_C > 1$.

In the second place, it should be noted that when using different alkaloid mixtures an alteration and often an inversion occurs in the R_C values of the single alkaloids. Furthermore, the presence of large quantities of some alkaloids influences the movement of the others.

In fact, it is not uncommon that an alkaloid mixture which appears to be simple in two-dimensional chromatography turns out to be much more complex when a large quantity of the same mixture is used, the same experimental conditions being maintained.

(b) Circular paper chromatography

In the case of very complex alkaloid mixtures it has been found that one-dimensional separation on paper is rendered still more difficult by the partial overlapping of a long series of alkaloids, which have R_C values that are very close to each other. In such cases the components come out much more clearly if the chromatogram is run on a small band only a few centimetres wide[27].

Better results are obtained by means of Rutter's circular paper chromatography in that clear-cut bands appear which are well differentiated from neighbouring bands.

This system, though very convenient for a preliminary investigation of alkaloid mixtures, has, however, the drawback that comparison data are very scanty in the literature. Nevertheless, it is extremely useful for selecting the most suitable separation solvents and for making a rapid examination of certain mixtures.

(c) Electrophoresis on paper

By means of electrophoresis on paper it is possible to separate numerous curarizing alkaloids from *Strychnos* species, as was shown for the first time by MARINI-BETTÒLO AND LEDERER[59].

A good separation can be obtained by adjusting the conditions such as the pH of the buffer. Since electrophoresis is based on principles that are quite different to those of paper chromatography, substances that have identical R_C values may often be well separated.

TABLE 3

ELECTROPHORETIC MIGRATION OF ALKALOIDS FROM *Strychnos* SPECIES AND CALABASH CURARES*

Alkaloids	*Displacement towards the cathode in 3 hours (in mm)*					
	pH 2.3	*pH 4.3*	*pH 6.4*	*pH 8.5*	*pH 10.5*	*pH 11.4*
Mavacurine	31	—	24	30	24	19
Fluorocurine	77	—	80	90	80	42
Alkaloid D	65	—	90	90	100	52
Calebassine	65	—	80	90	78	50
Curarine	106	98	110	108	—	64

* G. B. MARINI-BETTÒLO AND J. A. COCH FRUGONI, *Gazz. chim. ital.*, 86 (1956) 1326.

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For some alkaloids the mobility of the ions has been measured at various values of the pH; these mobilities are given in Table 3.

Electrophoresis on paper strips chiefly permits the rapid identification of alkaloid groups rather than the single components that are present (Fig. 2).

To this end the portable apparatus designed by MARINI-BETTÒLO AND COCH FRUGONI may be usefully employed in the field[67].

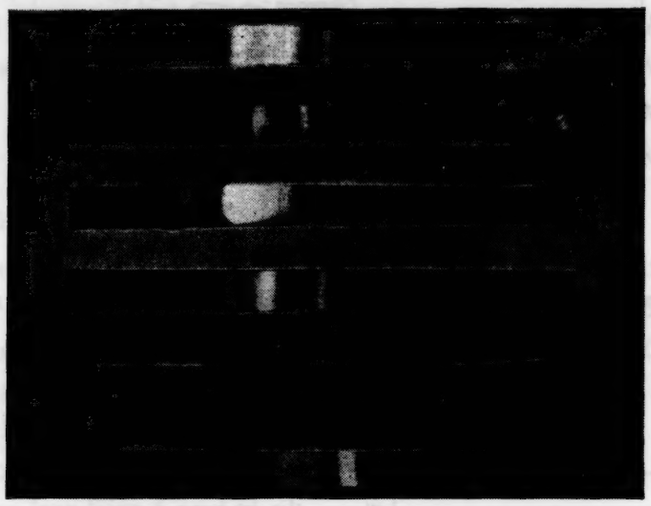

Fig. 2. Electrophoretic separation of *S. trinervis* alkaloids (fluorescent zones under U.V. light) (G. B. MARINI-BETTÒLO AND M. LEDERER, *Nature*, 174 (1954) 133).

(*d*) *Two-dimensional chromatography*

Two-dimensional chromatography is usually performed in the first direction with KARRER'S solvent "D" (Table 1) and in the second direction with KARRER'S solvent "C" (Table I).

TH. WIELAND suggests other solvents as reported in Table 1[94]. BOEKELHEIDE[20] found it more convenient to use a water–*n*-butanol mixture in one direction and

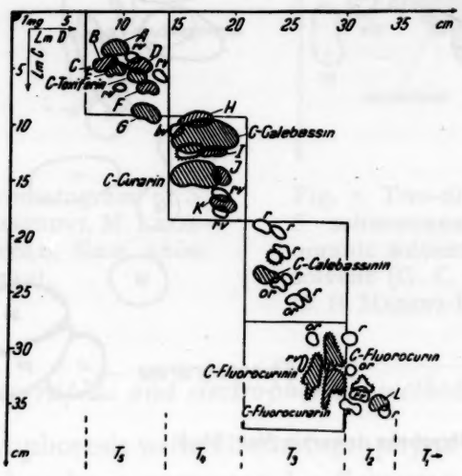

Fig. 3. Two-dimensional chromatogram of calabash curare alkaloid chlorides (H. SCHMID, J. KEBRLE AND P. KARRER, *Helv. Chim. Acta*, 35 (1952) 1864).

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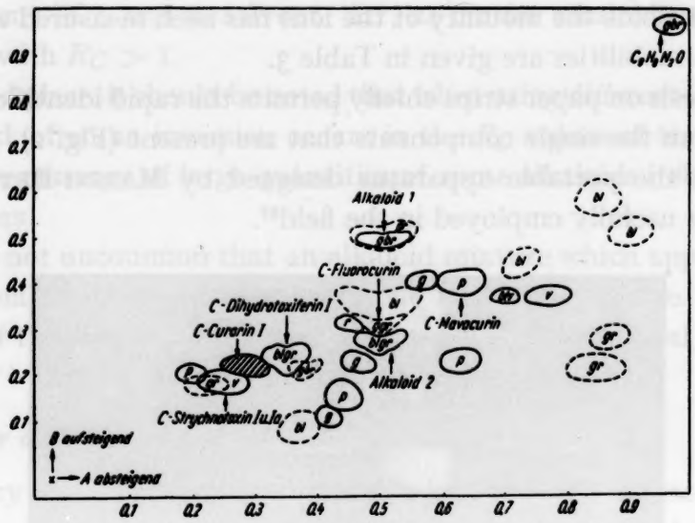

Fig. 4. Chromatogram of calabash curare alkaloid chlorides (TH. WIELAND AND H. MERZ, *Ber.* 85 (1952) 731).

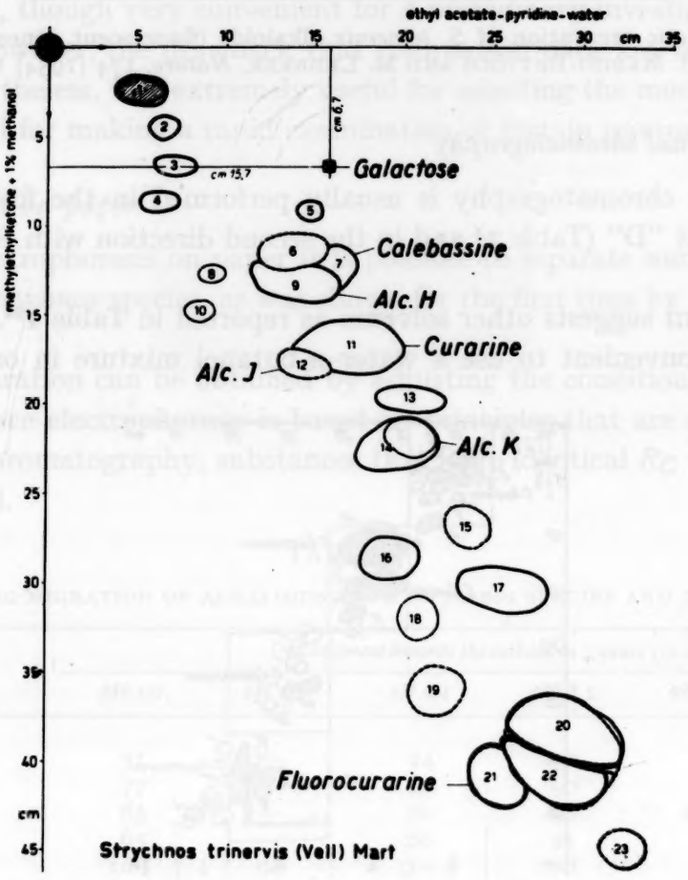

Fig. 5. Chromatogram of *S. trinervis* alkaloid chlorides (K. ADANK, D. BOVET, A. DUCKE AND G. B. MARINI-BETTÒLO, *Gazz. chim. ital.*, 83 (1953) 966).

KARRER'S solvent C in the other, thus avoiding the use of pyridine which is always troublesome.

For the determination of the relative displacement, curarine has generally been used as standard; other substances have, however also been taken, as previously stated, such as galactose and mavacurine[1,8].

Examination of the various chromatograms shows that the spots are ranged diagonally (Figs. 3, 4 and 5); this happens because the R_C values often have the same sequences in the two solvents. Here the same remarks apply which we made in the preceding pages with regard to reciprocal influences hampering the recognition of substances on the basis of R_C values only. This does not happen, for instance, with amino acids etc.

(e) Chromatography with aqueous solvents

To prevent a diagonal distribution of the alkaloids and thus facilitate the resolution of these mixtures, it is advisable to use other systems. To this end aqueous solvents have been suggested by CASINOVI, LEDERER AND MARINI-BETTÒLO[27].

Their study has been chiefly directed towards mixtures based on sodium acetate (see Table 1), which, in the case of *S. solimoesana* Kruk, produced a clear inversion of the spot sequence (see Figs. 6 and 7) obtainable with KARRER'S solvents C and D[27].

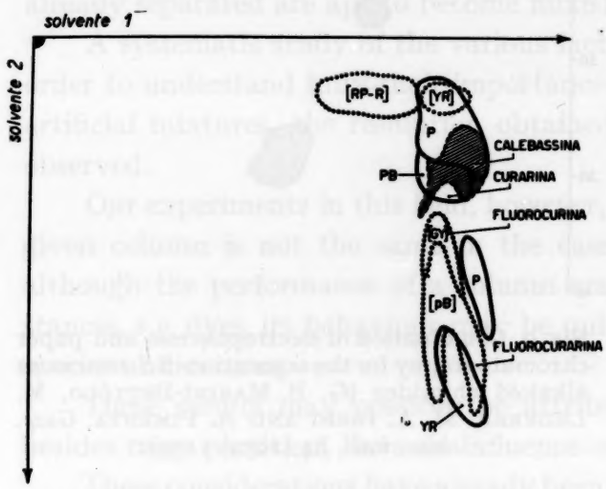

Fig. 6. Two-dimensional chromatogram of *S. solimoesana* alkaloids (G. C. CASINOVI, M. LEDERER AND G. B. MARINI-BETTÒLO, *Gazz. chim. ital.*, 86 (1956) 342).

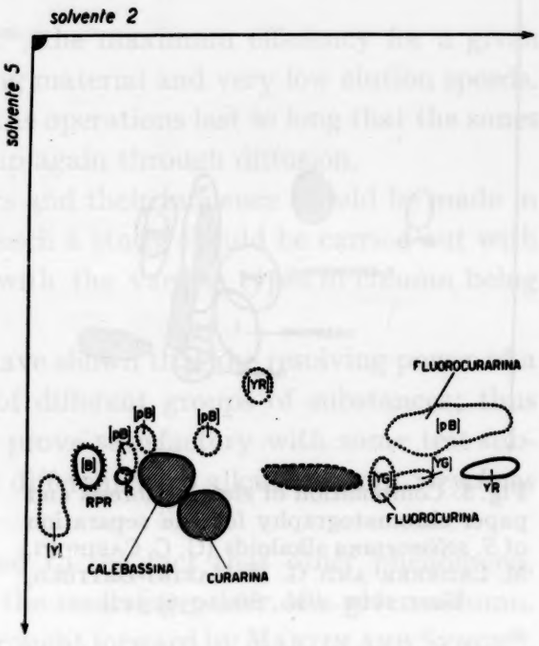

Fig. 7. Two-dimensional chromatogram of *S. solimoesana* alkaloids. First direction: organic solvent; second direction: aqueous solvent (G. C. CASINOVI, M. LEDERER AND G. B. MARINI-BETTÒLO, *Gazz. chim. ital.*, 86 (1956) 342).

(f) Combination of chromatographic and electrophoretic methods

By combining paper electrophoresis with chromatography, a very good resolution may be realized. This system has been suggested by CASINOVI, LEDERER AND MARINI-BETTÒLO[27] (see Fig. 8); electrophoresis should be used first and chromatography

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afterwards, and in the former a volatile electrolyte buffer (acetic acid or/and amine) should be employed in order to avoid interferences with the chromatographic stage.

In this case also the various spots are no longer arranged diagonally as in the case, for instance, with *S. tomentosa* alkaloids (Fig. 9).

These methods, however, have the drawback that diffusion occurs which makes the definition of the spots less precise. The same applies to two-dimensional paper electrophoresis when several buffers or a single one with different ionic forces are used[59,60].

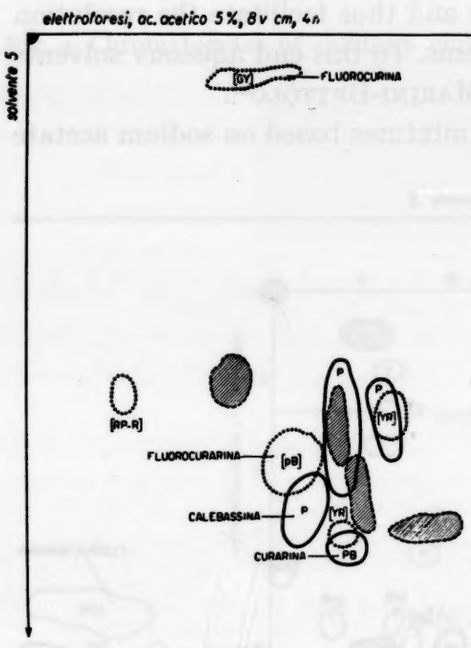

Fig. 8. Combination of electrophoresis and paper chromatography for the separation of *S. solimoesana* alkaloids (G. C. CASINOVI, M. LEDERER AND G. B. MARINI-BETTÒLO, *Gazz. chim. ital.*, 86 (1956) 342).

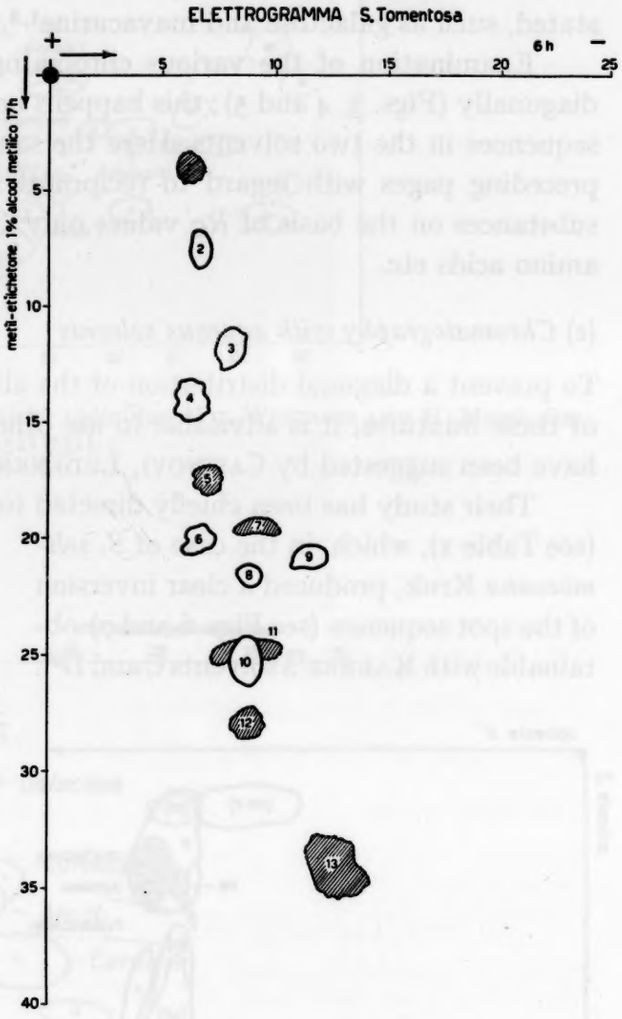

Fig. 9. Combination of electrophoresis and paper chromatography for the separation of *S. tomentosa* alkaloid chlorides (G. B. MARINI-BETTÒLO, M. LEDERER, M. A. IORIO AND A. PIMENTA, *Gazz. chim. ital.*, 84 (1954) 1155).

6. PREPARATIVE SEPARATION METHODS

(a) *Chromatography on absorption columns*

Chromatography on alumina columns has provided the first method for obtaining pure alkaloids either from curares or South American *Strychnos* species.

H. WIELAND, who worked with columns of alumina or Fuller's earth and utilized alkaloid reineckates or alkaloid perchlorates, was able to separate these alkaloids, whereby the first indication of the great complexity of these mixtures was obtained.

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SCHLITTLER AND HOHL[80] used alumina columns and eluted the reineckates of *S. diaboli* with acetone and methanol.

(*b*) *Partition chromatography on cellulose columns*

An improvement on the preceding method, which is, however, still being used for the separation of certain fractions, is the partition chromatography on cellulose columns, subsequently developed by SCHMID AND KARRER[85] and by TH. WIELAND[92]. In this separation KARRER'S solvent "C" is mainly used as in paper strip or in two-dimensional chromatography. BOEKELHEIDE[20] suggests the use of a specially prepared cellulose column and butanol–water as solvent. After a first separation by elution, slow-moving fractions were extruded and extracted separately.

Yet, even under these conditions, it is only by working with very large quantities of substances and discarding many fractions that appreciable quantities of pure alkaloids can be obtained; when employing small quantities it is extremely difficult to realize preparative separations that are of any use.

Indeed, various factors affect the resolving power of partition columns; the most important of these are packing, height, homogeneity, elution velocity, and weight ratio between substance and absorbent.

As pointed out by MARTIN AND SYNGE[69], the maximum efficiency for a given column is obtained by using very fine charging material and very low elution speeds. This is the main drawback to the method, as the operations last so long that the zones already separated are apt to become mixed up again through diffusion.

A systematic study of the various factors and their influence should be made in order to understand fully their importance; such a study should be carried out with artificial mixtures, the resolution obtained with the various types of column being observed.

Our experiments in this field, however, have shown that the resolving power of a given column is not the same in the case of different groups of substances; thus although the performance of a column may prove satisfactory with some test substances, *e.g.* dyes, its behaviour may be quite different with alkaloids from *Strychnos* species and curares.

These results may possibly be attributed to the fact that other phenomena, besides mere partition, have an influence on the resolving power of a given column.

These considerations have already been brought forward by MARTIN AND SYNGE[69], by CRAIG[29] and by HECKER[37].

The necessity of examining the other characteristics of a column is a logical consequence of the above remarks. For testing purposes, samples of the substances to be separated should be used; but since pure samples of *Strychnos* alkaloids are difficult to obtain, particularly of the high degree of purity required for all the tests necessary for the systematic study, the problem cannot yet be investigated fully.

Yet, despite the lack of numerical and systematic data for direct comparison of the various columns employed, such a comparison can still be made by using paper chromatograms of mixtures of alkaloids that can easily be obtained and chromatograms

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of the same mixtures, or others containing the same alkaloids on various columns.

For instance, the usual procedure when investigating the content of alkaloids in a given plant is to determine the composition of the mixture by common methods. Once the composition is known, the problem of separation on a preparatory scale arises.

As a rule, the available material is both scarce and of a complex composition, due to the great number of alkaloids and the closeness of their R_C values; therefore, an efficient resolution is indispensable, also from a quantitative point of view.

A comparison between the resolving power of a strip of paper and column of

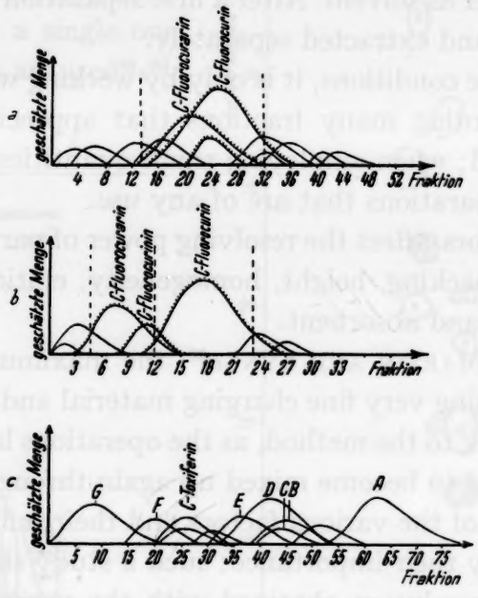

Fig. 10. Separation diagrams of calabash alkaloid chlorides (H. SCHMID, J. KEBRLE AND P. KARRER, *Helv. Chim. Acta*, 35 (1952) 1864).

equal length shows that the efficiency of the latter is very small, even when the charging density of the two is about the same and the weight ratio between mixture and cellulose is the same.

In their work on calabash curarizing alkaloids SCHMID AND KARRER[85] claim that the resolving power of the cellulose column coincides quantitatively with that of the corresponding paper chromatograms, but they only give schematic diagrams on the subject (Fig. 10).

A closer examination of these results shows, for instance, that in the T_5 group of the NIII fraction, alkaloids A (R_C: 0.55) and G (R_C: 0.73) are present together with other alkaloids, and that for their separation, contrary to expectation, many chromatograms using both solvents were necessary in order to secure complete resolution. Similar examples may be found in the work of the above-mentioned authors[85].

In the case of *S. melinoniana* alkaloids, BÄCHLI, VAMVACAS, SCHMID AND KARRER[8] give a diagram (Fig. 11) of the column chromatography of a mixture of at least 14 alkaloids, but the graph (see Fig. 11) shows only two well-separated maxima. Evidently, the resolving power is not high. As a further confirmation, reference may be

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made to the work of CASINOVI[26], who, when separating a relatively simple mixture of *S. amazonica* alkaloids, proved the inefficiency of a column whose resolving power was considerable for dye mixtures; consequently he had to resort to counter-current distribution in order to obtain satisfactory separations.

In an investigation of the fractionation of *S. macrophylla*[38] alkaloids by means of a cellulose column under normal conditions, it was found, by subjecting the fractions

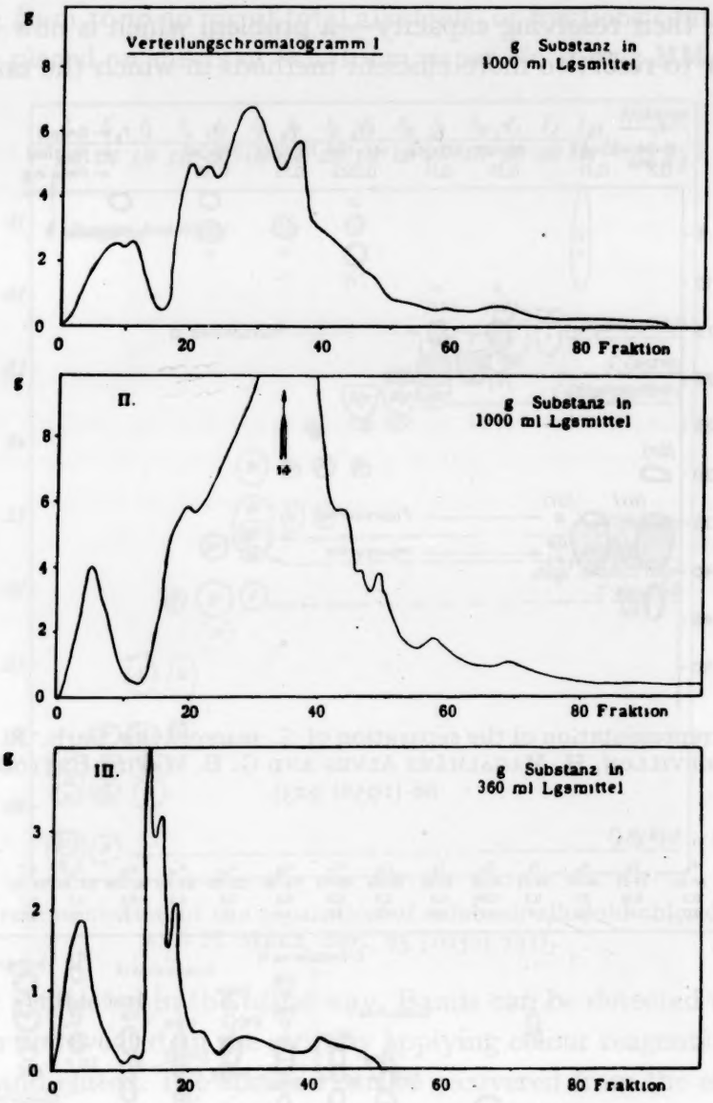

Fig. 11. Separation diagrams of *S. melinoniana* Baill. alkaloid chlorides (E. BÄCHLI, C. VAMVACAS, H. SCHMID AND P. KARRER, *Helv. Chim. Acta*, 40 (1957) 1181).

to paper chromatography, that the fractionation is fairly satisfactory for those alkaloids (Mavacurine – Macrophylline B or Macrophylline A and B) whose R_C ratio is very high, but that it is practically *nil* for alkaloids whose R_C values are very close together, *e.g.*, Fluorocurarine–Mavacurine, which on a paper strip of equal length would be easily separated (Fig. 12).

When the mixture consists of a great number of alkaloids, whose R_C values are

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very close together, as is the case with *S. solimoesana* Kruk., the cellulose column can only perform a preliminary separation into various groups and the isolation of small quantities of the single components is only possible by means of repeated chromatography on paper sheets[63].

The same considerations apply to alkaloids from *S. guianensis*[64] (Fig. 13) and *S. subcordata*[76], and to WIELAND's calabash curares (Fig. 14).

From what has been said about columns it follows that it is necessary on the one hand to improve their resolving capacity—a problem which is now being studied—and on the other to resort to more efficient methods in which the undesirable effects

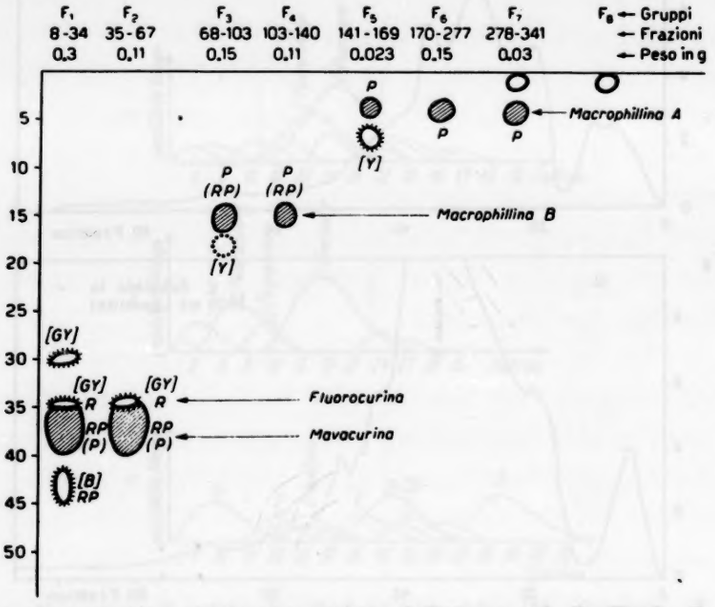

Fig. 12. Schematic representation of the separation of *S. macrophylla* Barb. R. alkaloid chlorides (M. A. IORIO, O. CORVILLON, H. MAGALHÃES ALVES AND G. B. MARINI-BETTÒLO, *Gazz. chim. ital.*, 86 (1956) 923).

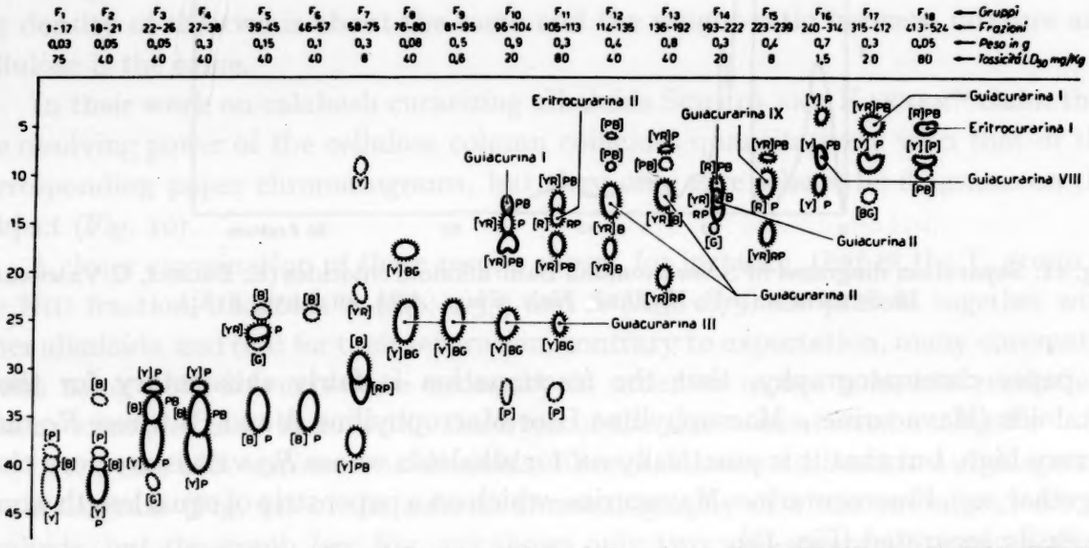

Fig. 13. Schematic representation of the separation of *S. guianensis* Aubl. Mart. alkaloid chlorides (G. B. MARINI-BETTÒLO AND M. A. IORIO, *Gazz. chim. ital.*, 86 (1956) 1305).

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of powdered cellulose are avoided, such as column electrophoresis and counter-current distribution, the first results of which seem to be rather promising.

(c) Preparative separation on paper sheets

Whenever the resolution of mixtures on cellulose or alumina columns is found to be difficult, one may resort to paper sheet chromatography since it allows the isolation of small quantities of pure alkaloids.

To this end, from 10 to 30 mg of total alkaloids, or fractions containing a number of alkaloids, are placed on sheets of Whatman paper No. 1 or 3 MM along a line and

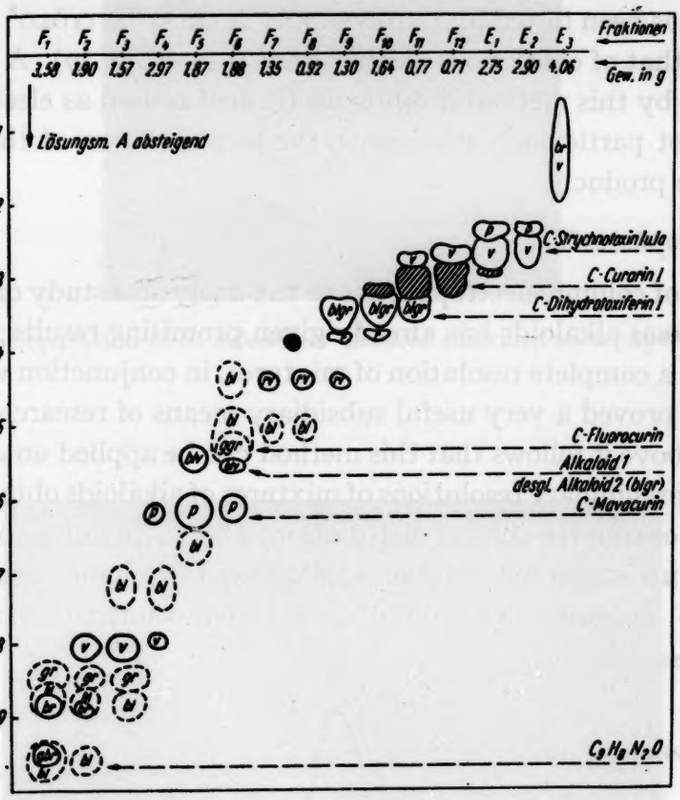

Fig. 14. Schematic representation of the separation of calabash alkaloid chlorides (TH. WIELAND AND H. MERZ, *Ber.*, 85 (1952) 731).

chromatography is effected in the usual way. Bands can be detected under a Wood's lamp or they can be revealed on the strip by applying colour reagents. The bands can then be cut out and eluted. The alkaloid can be recovered from the eluate and again subjected to chromatography, until a fraction is obtained that is chromatographically pure.

In the case of very complex mixtures with strongly differing R_C values, after cutting out the bands whose resolution appears satisfactory, another sheet of paper should be used and chromatography continued until a satisfactory resolution of the other bands is reached.

This method has been successfully employed with mixtures of alkaloids from *S. solimoesana*[63], *S. guianensis*[62] and *S. subcordata*[76]; numerous pure alkaloids can be obtained in this way (Fig. 16).

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(d) Band electrophoresis

Besides band chromatography, it may occasionally be advantageous to carry out a band electrophoresis in order to separate certain components. The fractionation of *S. guianensis* alkaloids may serve as an example of this point[64].

Since electrophoresis brings about a considerable diffusion of the bands, it is not advisable to repeat the operation, as is the practice in chromatography; instead electrophoresis should be followed by chromatography of the eluate on a paper sheet.

(e) Continuous electrophoresis

A method that has been tested by LEDERER AND MARINI-BETTÒLO* with *S. guianensis* and *S. froesii* is that of continuous electrophoresis (see Fig. 17). A good fractionation can be obtained by this method if dilute acetic acid is used as electrolyte; the results are, however, not particularly good since the long duration of the operation causes alteration of the products.

(f) Column electrophoresis

The application of column electrophoresis to the analytical study of mixtures of South American *Strychnos* alkaloids has already given promising results; although by itself it does not yield a complete resolution of mixtures, in conjunction with paper chromatography it has proved a very useful subsidiary means of research.

From the above it follows that this method can be applied on a preparative scale, particularly for preliminary resolutions of mixtures of alkaloids obtained from partition chromatograms or counter-current distribution. The results of a considerable number of preliminary investigations on the application of column electrophoresis to the purification of *S. amazonica* alkaloids obtained from counter-current distribution, are

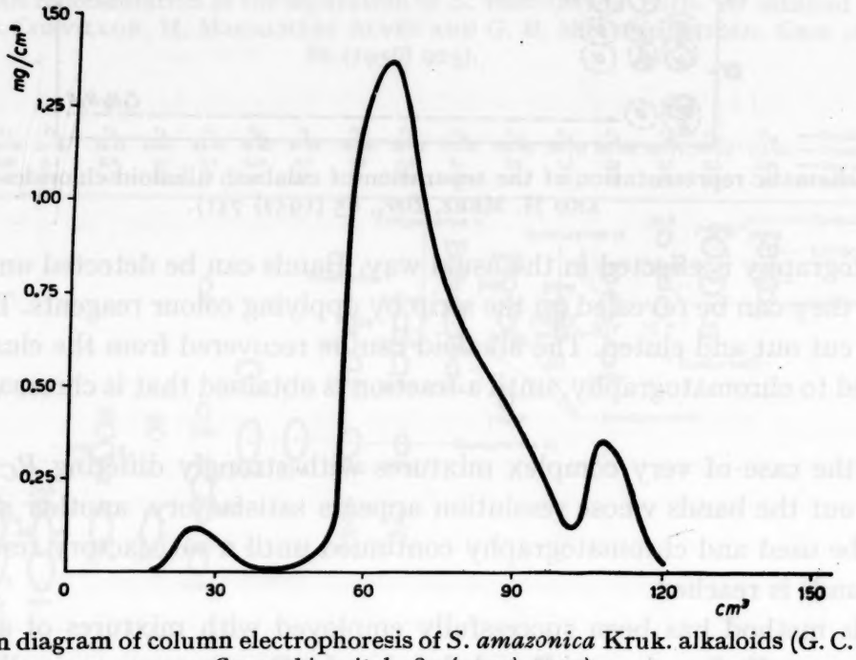

Fig. 15. Elution diagram of column electrophoresis of *S. amazonica* Kruk. alkaloids (G. C. CASINOVI, *Gazz. chim. ital.*, 87 (1957) 1457).

* Unpublished results.

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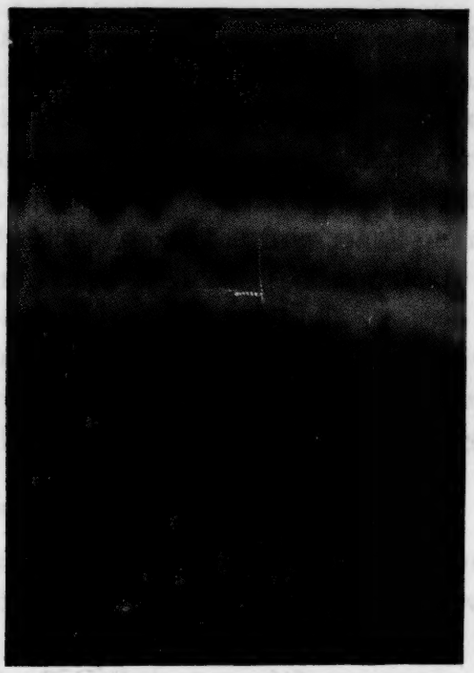

Fig. 16. Separation of *S. subcordata* alkaloid chlorides on a paper sheet.

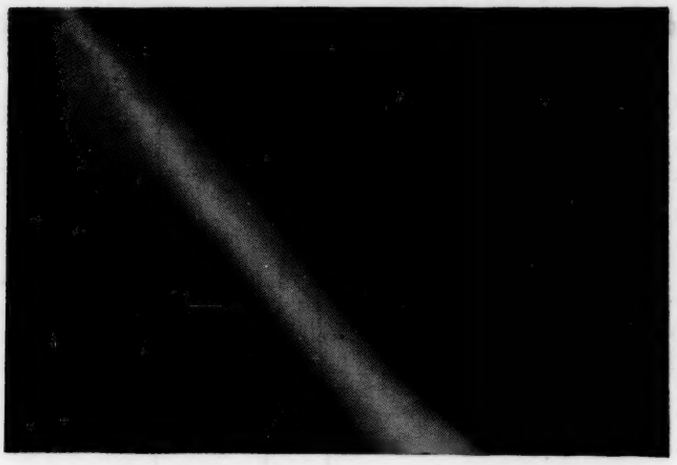

Fig. 17. Continuous electrophoresis of *S. froesii* Ducke alkaloid chlorides (M. LEDERER AND G. B. MARINI-BETTÒLO, unpublished results).

given in Fig. 15. This figure shows the elution curves of an electropherogram of crude alkaloids, which was obtained on a column of 2 × 100 cm, a potential of 800 V being applied for 14 hours and 2% acetic acid being used as electrolyte[26].

If the experimental data are plotted in a co-ordinate system in which the number of c.c. of eluate are taken as abscissae and the concentrations as ordinates, it will be seen how the principal alkaloid is separated from small quantities of other alkaloids, which separation could not have been effected so quickly by partition chromatography or counter-current distribution.

The aim of current studies on this subject is to establish whether this method can be applied not only to purification, but also to the preliminary fractionation of groups of substances that are to be subsequently separated by other methods.

(g) *Counter-current distribution*

Counter-current distribution is a highly efficient quantitative mehod, which can also be used on a preparative scale. It has numerous advantages over partition chromatography, such as:

1. The possibility of checking the course of the separation at any time by chromatographing small samples.

2. The absence, thanks to the discontinuity of the process, of diffusion phenomena, which in chromatography might affect the efficiency of the method in case of interruption of the flow in the column or long duration of the process.

3. The absence of irreversible adsorption phenomena, which on columns bring

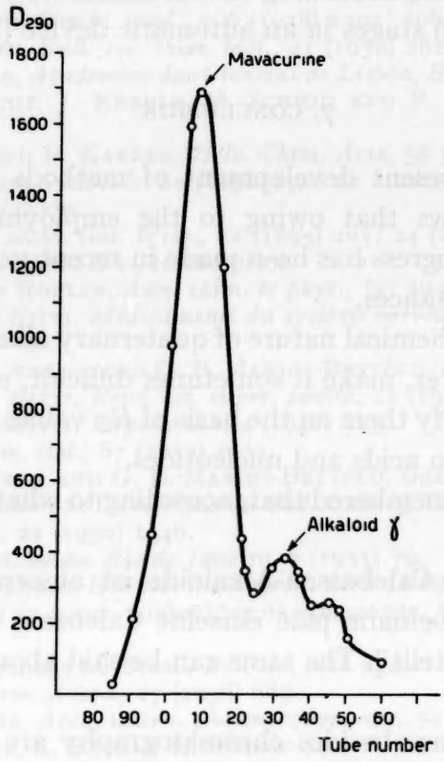

Fig. 18. Distribution diagram of *S. parvifolia* alkaloids in counter-current distribution (solvents: butanol–water).

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about a loss of substance and hamper successful performance, as well as the absence of all wall effects, which reduce the column's resolving efficiency.

The disadvantages lie in the complexity of the equipment, which is larger than in chromatography, the long duration of the process and the fact that emulsions are easily formed, which increases the time of operation.

CASINOVI[26] applied this technique to the separation of a fairly simple alkaloid mixture from *S. amazonica*. The apparatus had 15 tubes, the solvents were methyl ethyl ketone–water, and the operation was carried out with 1 g of total alkaloids. After 14, 18, 42 and 56 stages, the process was followed by chromatography. Using the same alkaloid mixture, and employing butanol–water and an apparatus with 25 tubes on the basis of data resulting from previous operations, it is possible to resolve almost entirely the mixture of *S. amazonica* alkaloids.

A third example of the application of this technique is the purification of an alkaloid that shows an intense yellow-green fluorescence under U.V. light. Owing to the low partition coefficient, the distribution was continued for 400 stages with the recycling method.

Once stage 400 had been reached, the distribution curve was plotted from measurements of the fluorescence under U.V. light: the resulting curve clearly proves that the product is homogeneous.

These tests, which up to the present have been carried out with a small apparatus have given interesting results. It may be assumed that by employing multi-tube apparatus the separation of two or more alkaloids whose partition coefficients are close together, will also be possible and that good results will be obtained, as is shown by diagrams obtained with 300 stages in an automatic device (Fig. 18)*.

7. CONCLUSION

An examination of the present development of methods for separating *Strychnos* quaternary alkaloids, shows that owing to the employment of chromatographic techniques considerable progress has been made in recent years in the separation and identification of these substances.

The peculiar physico-chemical nature of quaternary alkaloids and the complexity of natural mixtures, however, make it sometimes difficult, even nowadays, to obtain pure products and to identify them on the basis of R_C values, a difficulty not met with in the case of sugars, amino acids and nucleotides.

In fact, it should be remembered that, according to what SCHLITTLER AND HOHL[80] wrote some years ago:

"die Bearbeitung der Calebassen-Alkaloide ist ausserordentlich schwierig; sie krankt auch daran, dass beinahe jede einzelne Calebasse bei der Aufarbeitung ein gesondertes Probleme darstellt". The same can be said about the *Strychnos* bark and root alkaloids.

Today, other techniques besides chromatography are available, such as zone-

* Unpublished results.

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electrophoresis and counter-current distribution, which are based on different principles and offer new possibilities.

Moreover, it is worth noting that further progress is being made in the field of fractionation by combining various chromatographic techniques; examples are the separation of *S. toxifera* alkaloids where partition chromatography and chromatography on alumina are alternately employed[4], or the separation of alkaloids from *S. guianensis*[64] and *S. amazonica*[26] by combining chromatographic methods with electrophoresis and counter-current distribution.

These methods warrant our belief that it will be possible to resolve many mixtures that could not be fractionated up to the present. We are also of the opinion that here as elsewhere the necessity becomes ever greater of utilizing all available methods of separation.

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CHROMATOGRAPHY OF STEROLS, STEROIDS, AND RELATED COMPOUNDS

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I. INTRODUCTION

The various chromatographic methods, at first adsorption chromatography, and then later partition chromatography, have today become indispensable aids in the isolation, separation and determination of these substances. There is no need at this point to elaborate on the supreme importance and great usefulness in both isolation and synthesis of these methods of separation, which can be employed both for preparative purposes and also for qualitative and quantitative analysis. Examples will be found later, in the specific sections. Suffice it to note that suitable combinations of adsorption- and partition chromatography enable one to separate almost all possible mixtures in the most gentle way, without appreciable losses, and by purely physical means, employing no chemical reactions whatever. This is particularly important in the handling of labile compounds, or those whose properties are not yet known. The combination of chromatographic methods with chemical reactions can, however, be advantageous in the separation of certain other complicated mixtures.

All chromatographic methods with high resolving power require in principle only simple apparatus and simple procedures, which lend themselves in special cases to a very high degree of automation. Batch processing is no disadvantage in laboratory work in the field of steroids, since quantities from fractions of a microgram up to about 100 grams can be handled in one operation. It is true of course that with large quantities a considerable consumption of solvents is to be expected. For the application of chromatography on a technical scale, *cf.* [1]*.

The general theory of adsorption- and partition chromatography, and the apparatus required have already been discussed in detail in other places [2–13, 80]. Only equipment specially adapted to the handling of steroids will be mentioned here. It is the purpose of this review to describe in detail the methods which have proved best in practice, to present typical examples, and to discuss the relationship between chemical structure and chromatographic behavior. The literature is covered to the beginning of 1958.

For practical reasons, we shall discuss first column chromatography (adsorption and partition) and then paper chromatography (essentially partition). Ion-exchange and electrophoretic methods can be omitted from this review, since the steroids are

* In this paper, reference numbers are given in square brackets.

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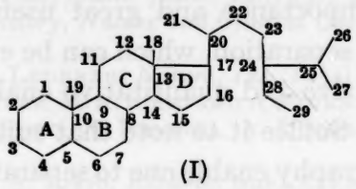
for the most part neutral substances. Even in the treatment of acid (estrogens, carboxylic acids) or basic (steroids alkaloids) compounds, such methods have scarcely been investigated, since even here the methods based upon adsorption and partition yield satisfactory results. Gas chromatography has not thus far proved practical, because of the low volatility of the steroids.

2. NOMENCLATURE, STEREOISOMERISM, AND STRUCTURAL FORMULAS OF TYPICAL STEROIDS

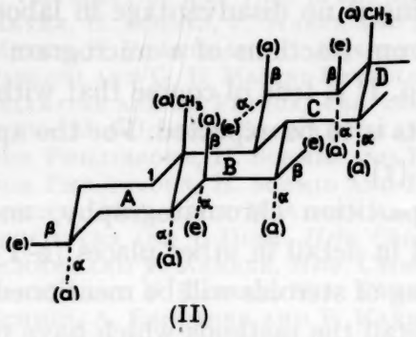
So far as the usual nomenclature involving chromatographic properties and methods of procedure is concerned, we remark only on the terms "polar" and "non-polar" which are often used in connection with adsorbents, solvents, and steroids. "Polar" here means hydrophilic, and "non-polar", hydrophobic or lipophilic.

The nomenclature of the steroids follows the internationally recommended system [14]. The following points are to be emphasized: Steroids are numbered according to formula I:

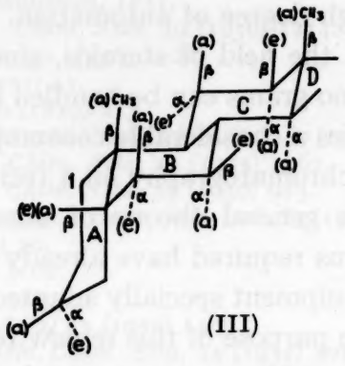


(I)

The decalin-like fusion of the hydrocarbon rings gives rise to a large number of stereoisomers. In naturally-occurring steroids the rings B and C are always *trans* to one another, and the rings C and D are usually *trans* (the *cis*-configuration is found only in the cardenolides and the bufadienolides). Rings A and B occur about as frequently in the *cis*-configuration as in the *trans*-configuration. The spatial formulas II and III give a clearer picture of these relationships. In order that the spatial



(II)



(III)

structure may be described unambiguously, the directions of the valences of the ring carbon atoms are referred to the angular methyl group at C_{10} [82]. This methyl group is by convention taken to lie *above* the plane of the four rings, and is represented by the solid line. This position is designated as the β-position; the opposite direction, below the plane, is the α-position (denoted by a dotted line).

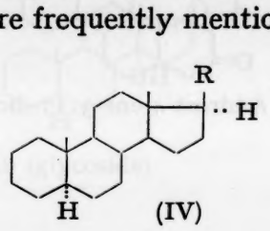
The C–H bonds in II and III are shown only in positions 3, 5, 6, 7, 11, 12, and 14. Bonds to these C-atoms are also designated as e (equatorial) or a (axial), to show the

References p. 186.

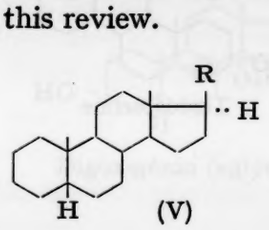
positions in space of the substituents bound to the rings. These spatial positions may be correlated with physical-chemical properties of the compounds [15, 16].

For example, hydroxyl groups in the equatorial position (and therefore radial to the axis of symmetry of the cyclohexane ring) are always found on partition chromatography to be more polar than those in the axial position (parallel to the symmetry axis). Therefore, depending upon the manner of joining of rings A and B, both the α-isomer (A and B *cis*, III) and the β-isomer (A and B *trans*, II) can have 3-hydroxy groups in equatorial (and hence, from a physical-chemical standpoint, equivalent) positions.

The following eight names (Table 1) will be used to designate the hydrocarbons IV and V, which are frequently mentioned in this review.



(IV)



(V)

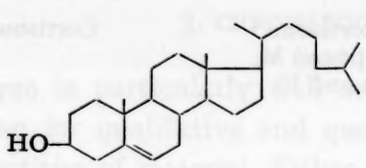
TABLE 1

	IV 5α-series	*V 5β-series*
R = H	5α-androstane	5β-androstane
R = $-C_2H_5$	5α-pregnane	5β-pregnane
R = $-CH(CH_3)CH_2CH_2CH_3$	5α-cholane	5β-cholane
R = $-CH(CH_3)CH_2CH_2CH_2CH(CH_3)_2$	5α-cholestane	5β-cholestane

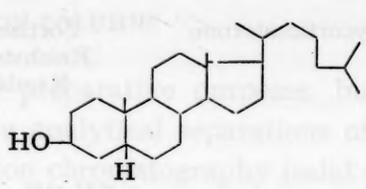
The side chain at C_{17} is usually in the β-position in the natural steroids. When it is not, one uses the designation 17α, *e.g.* 17α-pregnane.

Structural formulas of several typical representatives of the sterols, steroids and related compounds are given below, together with their trivial names. (The hydrogen atom at C_{14} is always in the α-position, if it is not specifically written in.)

Sterols

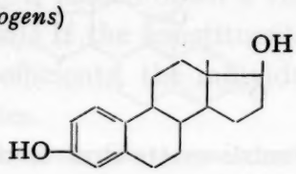


Cholesterol

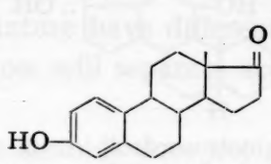


Coprosterol

C_{18}-Steroids (estrogens)



Estradiol-17β*



Estrone

* *Translator's note.* This substance was formerly called α-estradiol; the usage has persisted in some medical literature.

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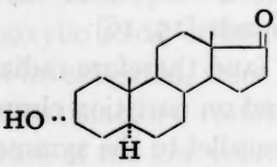
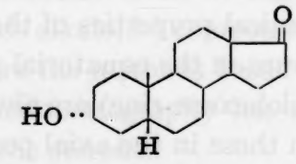
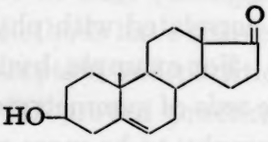
C_{19}-Steroids



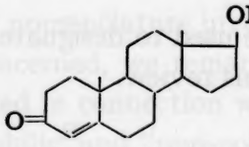
Androsterone



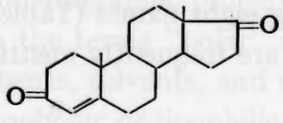
3α-Hydroxy-5β-androstan-17-one
(formerly etiocholanolone)



Dehydro*epi*-androsterone

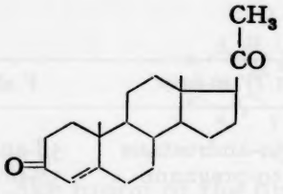


Testosterone

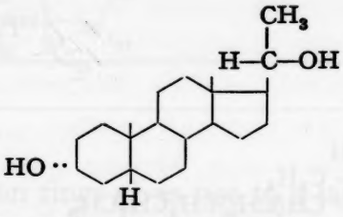


Androst-4-ene-3:17-dione*

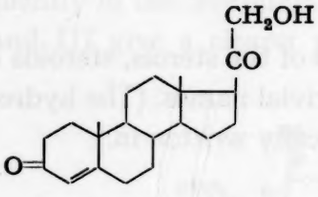
C_{21}-Steroids



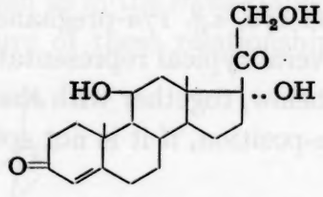
Progesterone



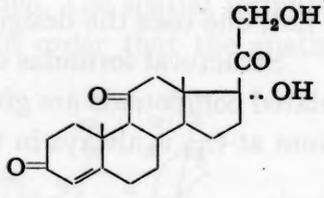
5β-Pregnane-3α:20α-diol
(pregnanediol)



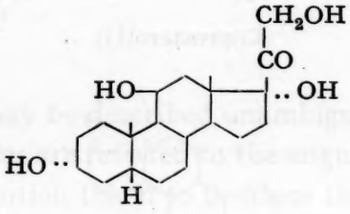
11-Deoxycorticosterone



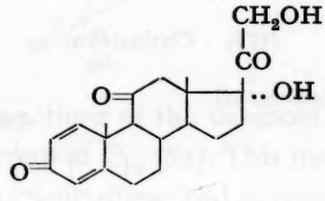
Cortisol (hydrocortisone,
Reichstein's compound M,
Kendalls compound F)



Cortisone



Tetrahydrocortisol,
3α:11β:17α:21-tetrahydroxy-5β-pregnan-20-one



Prednisone (1-dehydrocortisone)

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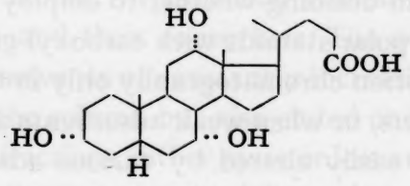
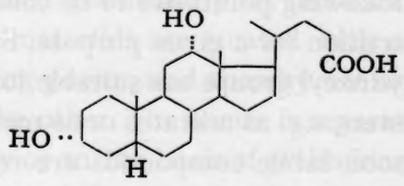
* *Translator's note.* The symbol Δ to denote the double bond is no longer used in formal nomenclature.

Bile acids

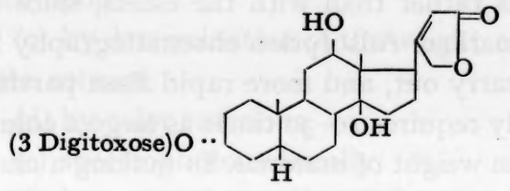


Cholic acid

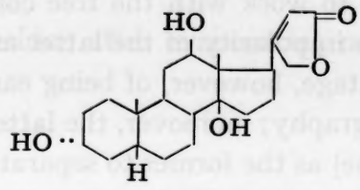


Deoxycholic acid

Cardenolides

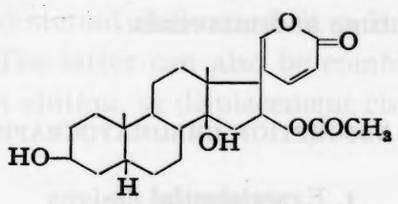


Digoxin (glycoside)



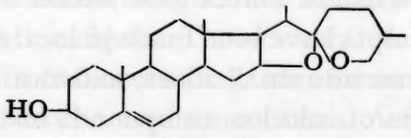
Digoxigenin (aglycone)

Bufadienolides



Bufotalin

Sapogenins



Diosgenin

3. CHROMATOGRAPHY ON COLUMNS

This technique is particularly well suited for preparative purposes, but is often employed also for qualitative and quantitative analytical separations of even the smallest quantities of material. Either adsorption chromatography (solid and liquid phases) or partition (between two liquids) may be used. The mixture, dissolved in a suitable solvent, is passed down a vertical column wetted with another solvent (the stationary phase). If the constituents of the mixture have different adsorption- or distribution co-efficients, the individual substances will separate according to their varying mobilities.

The structural- and stereo-isomerism of the steroids does not give rise to any great difference between the separating powers of adsorption chromatography on the one hand, and partition chromatography on the other. An exception is the isolated double bond, whose influence is somewhat greater in adsorption than in partition.

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The following points are to be considered in deciding whether to employ adsorption or partition for a given purpose. Strongly polar steroids with carboxyl groups or several hydroxyl groups are suitable for adsorption chromatography only in the form of their esters, *e.g.* as acetates or as methyl esters, or when weak adsorbents are used. Furthermore, labile compounds are relatively easily altered by various adsorbents, and it should also be borne in mind that most substances in adsorbed form are more sensitive to oxygen and to light than they are in solution.

These limitations do not apply to partition chromatography. Here it is actually preferable to work with the free compounds rather than with the esters, since the differences in polarity of the latter are less marked. Adsorption chromatography has the advantage, however, of being easier to carry out, and more rapid than partition chromatography; moreover, the latter usually requires 10–30 times as large a column (by volume) as the former to separate a given weight of material. In making a choice between the two methods one must therefore consider:

(1) Polarity and lability of the substance.

(2) Separating power.

(3) The consumption of time and materials.

A. ADSORPTION CHROMATOGRAPHY

1. Experimental designs

The method given by TSWETT for colored substances can in principle be used for colorless materials like the steroids. Direct observation of the individual zones is of course not possible, but attempts have been made to locate them by other means, *viz.*:

(1) Sectioning the column into small zones, and eluting them.

(2) Eliciting fluorescence of colorless compounds in the column by illumination with UV-light.

(3) Adding to the adsorbent fluorescent materials whose fluorescence is quenched at points where materials are adsorbed.

(4) Painting a narrow stripe of a suitable reagent (*e.g.*, $KMnO_4$) on the column after it has been pushed out.

(5) Co-chromatographing dyes with adsorption coefficients similar to those of the steroids being separated.

(6) Converting the steroids into colored derivatives, such as dinitrophenylhydrazones for ketones, azobenzoyl-*p*-carboxyl esters [17] for phenols, azoyl derivatives for steroids with reactive hydroxyl groups [18] or for bile acids [19, 21, 24].

(7) The possibility has recently arisen of converting steroids into radioactive esters with ^{131}I-*p*-iodobenzoyl chloride, for example, and determining their position on the column with a counter [20, 119].

Most of these methods entail so many disadvantages that they have made little headway. The method of choice, which is now used almost exclusively in the adsorption chromatography of steroids (and most other compounds, whether colorless or

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colored) is that of *fractional elution*, introduced particularly by REICHSTEIN and RUZICKA and their co-workers. The adsorbed materials are treated with a series of suitable solvents of constantly-increasing eluting power, and thereby not only separated on the column but also eluted one after the other. The eluate is separated into a series of fractions, either more or less arbitrarily or on the basis of preliminary experiments; alternatively, one follows the appearance of the dissolved, colorless material:

(a) by the schlieren effect,

(b) by the crystallization of the material on the delivery-tube tip when it is blown upon,

(c) by determination of the weight of the residue after evaporating or distilling off the solvent,

(d) by color reactions,

(e) spectrophotometrically,

(f) by measuring radioactivity, etc.

The most common method is probably the determination of weight. Further details will be given below, where the actual performance of experiments is described. Even in the case of colored steroid derivatives it is most advantageous by far to employ fractional elution. The latter can also be combined to advantage with the so-called method of gradient elution, or displacement chromatography.

2. Adsorbents

The most suitable adsorbents for the chromatography of steroids are homogeneous, hydrophilic materials like alumina, magnesium silicate, silica gel or silicic acid, or mixtures of the latter with kieselguhr. The activity of the adsorbents decreases in the above order. Magnesium carbonate [21], aluminum silicate [22, 23] or charcaol (hydrophobic) [25, 26] can also be used on occasion. A finer gradation of properties can be achieved by mixing adsorbents with different activities, or especially by stepwise activation or de-activation of an adsorbent. Partially inactivated preparations are often preferable to very active ones, since neutralization of the most active centers causes them to become considerably more homogeneous in their adsorptive power, thus showing less tendency toward tailing when the adsorbed substances are eluted, and hence giving, in the final analysis, a better separation.

Activation of hydrophilic adsorbents is usually achieved by heating (removal of water) and de-activation by the addition of definite quantities of water. The smaller the size of the particles, the better the adsorption. When the particles are very small, the speed of filtration drops; this effect can be counteracted by mixing the adsorbent with a weakly-adsorbing filler, like kieselguhr. The size of the particles is frequently expressed in terms of the mesh size, that is the number of holes per cm of a sieve which just passes the particles. A mesh size of 100 corresponds to a particle size up to about 0.05 mm. In English units the mesh size is expressed in holes per inch*.

* *Translator's note.* In English-speaking countries the mesh is frequently the number of holes per *square* inch.

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In selecting a suitable adsorbent one has to consider the polarity and the lability of the steroids being separated. Hydrophilic adsorbents adsorb a molecule more strongly the more polar groups it contains and the more unsaturated it is (especially when it contains conjugated double bonds). Weakly-polar compounds are therefore chromatographed on strong adsorbents, and strongly polar compounds on weak adsorbents.

Adsorbability increases in the order CH = CH, OCH_3, COOR, C = O, CHO, SH, NH_2, OH, COOH. The ratio between the number of C-atoms and the number of polar groups is of course also important; C_{19}-diols are for example more strongly adsorbed than C_{21}-diols. The adsorbability series is approximately reversed on hydrophobic adsorbents, so that the weakly polar compounds are most strongly adsorbed from organic solvents, and the strongly polar ones are most weakly adsorbed.

Alumina. Activated and standardized alumina can be obtained commercially (*e.g.*, from Merck, standardized according to BROCKMANN, basic and free-of-alkali). Equivalent preparations, which are very active, can be prepared by heating commercial pure aluminum hydroxide for three hours to 380–400° with stirring. Such preparations, which are very hygroscopic and rapidly take up water on standing in air, always contain free alkali, and give good separations. For certain purposes this alumina is too active or too alkaline, and causes condensations of ketones and aldehydes, splitting out of hydroxyl groups, etc. [27].

De-activation. De-activation is achieved by allowing the material to stand in air, followed by determination of the (decreased) activity by adsorption of selected dyes according to the procedure of BROCKMANN and SCHODDER [28]. (The levels of activity are denoted by a scale from I to V.)

This somewhat tedious procedure can be simplified by adding to the fully-active (water free) alumina in a glass container the amounts of water given in Table 2, and shaking until the mass becomes homogeneous (approximately 2 hours) and reaches the designated degree of activity [29].

TABLE 2

ACTIVITY LEVEL OF Al_2O_3 AS A FUNCTION OF THE WATER CONTENT [29]

Water added %	*Activity level Brockmann and Schodder* [28]	
0	I	highly active
3	II	
6	III	
10	IV	
15	V	weakly active

An activity level of II–III is frequently employed. Another simple method of de-activation is to equilibrate the alumina for about 5 days over a saturated solution of $NaBr.2H_2O$, which causes the adsorbent to take up 7–8% of water [30], or for three weeks over 58% sulfuric acid, changing the acid frequently until the concentration no longer varies [31]. It is also possible by thorough mixing of aluminum

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hydroxide preparations of different activities to obtain one with any desired intermediate activity. The adsorbent must always be stored in a well-closed container in order to keep the water content, and hence the activity, constant. For the same reason, the solvents used in chromatography must be anhydrous.

Alkali-free aluminum hydroxide is used for sensitive materials like aldehydes, ketones, lactones, easily-hydrolysable esters, etc. Such very weakly alkaline preparations (pH 7.5–8) can also be obtained commercially (*e.g.* from Woelm). To prepare them in the laboratory, one boils ordinary aluminum oxide repeatedly with distilled water until the filtrate becomes neutral. The precipitate is washed with methanol and re-activated in vacuo (10 mm) at 160–200°. Preparations activated at 200–210° are too active for many purposes, and a temperature of 180° is usually sufficient [27]. A stepwise de-activation like that described above can also be performed.

Alkaline alumina can of course be neutralized more rapidly with acid. A suspension in water is neutralized with dilute nitric acid, the product boiled with distilled water, and treated as described above. However, the product contains nitrate ions, which are not always innocuous. The use of hydrochloric, sulfuric, or acetic acids is rarely recommended.

Regeneration. If necessary, used alumina can be regenerated by repeated extraction with boiling methanol and water (with the addition of a little sodium hydroxide if necessary), washing with methanol, and heating. A faint coloring is of no consequence.

Magnesium silicates can be obtained commercially under this name (*e.g.* No. 34 of the Philadelphia Quartz Comp., Berkeley, Cal.) or under other designations (such as Florisil, 60–100 mesh, Floridin Co., Clarendon, Pa.). They are usually used after mixing with kieselguhr (1:1 or 2:1 magnesium silicate: kieselguhr). REICHSTEIN *et al.* [32] give explicit directions for preparing the mixture. The magnesium silicate, if it is not neutral in reaction, is suspended in water and treated with acetic acid until it is barely acid to litmus. It is then washed thoroughly on a Buchner-funnel with hot distilled water, until the washings become neutral, boiled twice with methanol, and finally dried at 200° (not in vacuo). Kieselguhr (*e.g.*, Celite, Johns Manville International Corp., New York) is extracted with boiling water and then with boiling methanol, and dried at 110° (no vacuum). Two parts by weight of magnesium silicate and one of kieselguhr are thoroughly mixed and used as the adsorbent "silicate mixture". SAMUELS *et al.* [33, 34] use a 1:1 mixture of magnesium silicate with celite or florisil. The adsorbent is washed for several hours, first with 95% ethanol, and then with absolute alcohol. After drying for 24 hours at 120° C it is activated at 600° C for 4 hours, and stored in the dry state over silica gel. After it has stood for several days, it is best to dry it again for 24 hours at 120° C if the humidity is high.

Silica gel (silicic acid gel). Good commercial products can be used directly as adsorbent, if, for example, one part suspended in 100 parts of water gives a clear and neutral filtrate. If the filtrate is acid, it is necessary to wash with distilled water until it becomes neutral. For special purposes the silica is pre-washed with organic solvents until they no longer contain any residue. In order to obtain preparations always

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having the same activity it is advantageous to heat 24 hours at 120° before use. This causes complete activation; the material is cooled in a desiccator. Davison silica gel (through 200, No. 923 or 922—fine grained— Davison Chem. Corp., Baltimore, Md.) is often used. Mallinckrodt silicic acid (Ramsay and Patterson [125]) is also suitable. Certain steroids are partly destroyed or altered by silica gel which is entirely free of water [120].

De-activation. According to CAHNMANN, partially inactivated preparations [35, 120] have recently proved valuable in chromatography of polar steroids, like, for example, the corticosteroids [36]. Like alumina, the silica gel is inactivated by adding water. The gel is first activated completely (24 h at 120°) and then treated with the desired quantity of water and mixed well in a closed container for several hours, until it becomes homogeneous. A calibration in terms of activity levels is unnecessary; the water content is simply reported. The latter should be between 5–25%. Preparations with 15–20% of water gave better separations of corticosteroids, with less tailing, than did fully activated silica gel [36]. Mixtures of water-free and very wet silica gels yield after a few hours without further treatment homogeneous preparations with intermediate water contents. Mixing silica gel with kieselguhr increases the filtration rate.

Regeneration [37]. Used silica gel is boiled with 5–10 volumes of 1% sodium hydroxide for ½ hour. If the mixture is not strongly alkaline to phenolphthalein the necessary amount of 1% alkali is added. The gel is filtered while hot, washed three times with distilled water, boiled for ½ hour with 3–6 volumes of 5% acid. It is then filtered, washed with distilled water until the filtrate becomes neutral, dried at 120° and screened through a No. 80 sieve.

Activated charcoal. There are many available commercial preparations of this adsorbent, which is, however, not much used in the chromatography of steroids (*e.g.*, Darco G-60, Mallinckrodt, Activated N.F.). The charcoal is used as such, mixed with kieselguhr, or after washing with dilute hydrochloric acid and potassium cyanide [38, 25, 26, 39].

3. Solvents

The adsorption strength depends not only upon the adsorbent and on the material adsorbed, but also upon the solvent. The more firmly the solvent is bound to the adsorbent, the more it competes with the solute for the active centers of the adsorbent, and the less firmly is the solute bound. The so-called elutotropic series of solvents given below means therefore that the solvents are successively more strongly adsorbed to hydrophilic adsorbents, that they elute adsorbed solutes increasingly well, and that the solutes are therefore decreasingly adsorbed. The series runs: pentane, petroleum ether, carbon tetrachloride, benzene, ether, methylene chloride, chloroform, acetone, ethyl acetate, propanol, ethanol, methanol, water, acetic acid or pyridine.

The degree of binding can be regulated very exactly by suitable choice of adsorbent and solvent, and the best medium for optimal separation thus discovered. The column is usually prepared with the solvent in the above series which precedes

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that in which the mixture to be separated is dissolved. When charcoal, a hydrophobic adsorbent, is used, the elution series is approximately the reverse of that for hydrophilic adsorbents.

All organic solvents should be dry and freshly-distilled. Several of them are available commercially "for chromatography". *Cf.* also [391].

Pentane and *petroleum ether* (b.p. 60–80° or 80–100°), *carbon tetrachloride*, *methylene chloride* and *acetone* are dried with calcium chloride, filtered, and distilled.

Benzene is shaken or stirred several times with concentrated sulfuric acid (a vibrator is very useful for this operation) washed with water until neutral, and distilled over phosphorus pentoxide or sodium wire.

Ether is washed with ferrous sulfate solution and with water, dried with calcium chloride, and distilled over sodium wire.

Chloroform is always sold with the addition of alcohol (1%) as stabilizer, and can be used in this form, but it is better to wash with water, dry carefully with calcium chloride, filter, and distil. This treatment removes only part of the alcohol, but it suffices to diminish the eluting power quite considerably compared to that of untreated chloroform. Completely alcohol-free chloroform is very unstable and is not to be recommended for chromatography.

Ethyl acetate must be freed of acid and alcohol by washing with soda solution and with water. It is dried with calcium chloride and distilled.

Several rather similar series of solvents are listed below. They have been extensively tested with various adsorbents, and have proved quite suitable. For *alumina:* Pentane, petroleum ether, benzene, ether, chloroform, methanol [27]. One should not proceed directly from one solvent to the next, in order to avoid cracks in the column and too great discontinuities in the eluting action. One adds rather perhaps 10% of the solvent to follow, increases this stepwise as necessary to 20, 30, and 50% and then goes over to the next pure solvent. It is sometimes necessary to make the steps much smaller, *e.g.*, 0.5, 1, 2 and 5%; this is particularly advisable on going over to ether and methanol (*cf.* the examples below). It should be noted that many solvents heat up on mixing; the mixtures must therefore be prepared ahead of time so that they are at column temperature when used. One can also proceed by starting with carbon tetrachloride, benzene, or chloroform, continuing by adding a very small amount of a highly-polar solvent, *e.g.*, 0.1% of methanol or ethanol, and slowly increasing this to approximately 5–10%. For this type of chromatography, using only two different solvents, the so-called gradient elution method is especially suited, the proportion of the polar solvent being increased continuously, rather than stepwise. Acetone and isopropyl ether are not to be used on alumina because they are relatively easily condensed to rather non-volatile compounds. One should also be aware, when using methanol, that alumina is slightly soluble in it.

Similar solvents are used for *magnesium silicate, e.g.*, petroleum ether–methylene chloride–acetone–ethanol, petroleum ether–benzene–ether–ethyl acetate, benzene–chloroform–ethanol, or chloroform–ethanol. Here too, and also in the case of silica gel, the solvent transitions should be made stepwise.

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For *silica gel* the series petroleum ether–benzene–ether–ethyl acetate–acetone–methanol, benzene–methylene chloride–chloroform–acetone–methanol, chloroform–ethanol or chloroform–*tert.*-butanol have proved satisfactory. Silica gel columns appear transparent with oxygen-free solvents and opaque with oxygen-containing ones. They must be handled carefully, otherwise cracks appear easily.

4. Apparatus

The customary simple set-ups can be used without modification for steroids, so that this subject need not be discussed further. One can usually get along without cooling- or heating jackets. Tubes of the dimensions given by REICHSTEIN AND SHOPPEE [27] for various quantities of alumina (Table 3) and with sealed-in sintered glass plates or with sealed-on stopcocks are very suitable. One should choose a tube of such dimensions that it is less than half-filled with alumina. For special purposes long, thin tubes are also used, or the tubes may be filled higher with the adsorbent and bear at the top a device for adding a corresponding volume. Microcolumns can be made from micropipettes which have been cut in two, or from microdropping tubes with constricted bottoms and widened tops (no stopcocks are needed). Grease is of course not used for any part which comes into contact with solvents.

TABLE 3

DIMENSIONS OF CHROMATOGRAPHY TUBES [27]

g Al_2O_3	*Internal diameter of tube mm*	*Tube length mm*	*Bore of stopcock mm*
1	8	110	3
2	10	130	3
4	13	160	4
8	16	200	4
15	20	250	4
30	25	300	6
60	32	400	6
125	40	500	6
250	50	600	8
500	65	750	8

5. Preparation of the column

The following procedure has proved suitable for preparing a uniform, air-free *column of alumina* [27]. If the tube has no sintered glass plate one introduces a wad of glass-wool, followed by a snug-fitting porcelain filter plate (or a wire-gauze) and a circular filter which fits exactly. The cock is closed, and the tube filled with the solvent to be used. When all the air bubbles have been removed a thin layer of very clean sand is added, and covered with a round filter. The tube is cautiously tapped while the alumina is being added in a fine stream. The adsorbent is allowed to settle, the

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cock is opened, and the solvent drawn off. The tube is repeatedly refilled with solvent, until the adsorbent no longer shows any tendency to settle. During this process the alumina must *always* remain covered with solvent. The column is covered with a circular filter, wadding, and sand or glass beads, in order to protect against stirring up of the adsorbent. The solvent is now allowed to drain off until it just covers the alumina, and the column is ready for use. Slight positive pressure can be used to speed the preparation of the column and for the chromatography. Filtered compressed air or gas from a tank with a reducing valve may be used or a simple pressure device using a column of water may be set up.

When the column has a sintered glass plate the procedure is similar, but simpler. The alumina is placed directly on the plate. The column should be free of gas bubbles, pores, and cracks. Negative pressure (suction on the lower end) should not be employed since this can lead to cracks and gas bubbles in the presence of volatile solvents. (For the special procedure in which the activity of the alumina is adjusted by drawing air through, *cf.* [40].)

The ratio of the diameter of the column to its height (not the height of the tube) varies within wide limits from about 1:2 to 1:20, and is in special cases occasionally even smaller. Usually, however, the ratio is between 1:4 and 1:8. The ratio of material to adsorbent (alumina) can also range from around 1:20 to 1:2000, and is usually about 1:30. Ratios of 1:100 to 1:2500 are in practice used only for special routine purposes like, for example, the quantitative analysis of very complex mixtures of urinary steroids [31, 40–42]. The ratio refers not to the total extract to be chromatographed, but usually only to the steroid content. If one uses suitable eluants one thereby achieves a very constant elution volume for the various steroids, in spite of the presence of large quantities of impurities.

The procedure described above can also be used for the preparation of ***magnesium silicate*** and ***silica gel columns***. For these adsorbents, however, it is better to pack the bottom of the tube with a uniform layer of wadding alone, add the adsorbent (in the form of a slurry in the solvent) at a single pouring, if possible, keeping the cock open, and allow the adsorbent to settle as described before. The adsorbent is then covered with a protective layer. (This procedure can also be used for alumina.)

Columns with the same dimensions can be used for silica gel or magnesium silicate as for alumina. The proportion of material to adsorbent is usually 1:30 to 1:60, although for special purposes considerably larger amounts of adsorbents are used, up to 1:200 for magnesium silicate [43, 34] and 1:1000 for silica gel [44]. In the case of one micro-determination of blood steroids, the adsorbent has actually been used in 100,000-fold excess over the steroids [45].

6. Execution of the chromatography

General example using alumina [27]. The mixture to be separated is made up as a 1–5% solution in that solvent which stands as near to the head of the elutotropic series as the solubility will permit (*e.g.*, 1 g in 100 ml petroleum ether). The solution

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is allowed to percolate through the column (30 g alumina) either with or without a slight excess pressure, and the filtrate is collected and labelled Fraction 1. Further 100 ml portions of the same solvent are allowed to pass through, and collected as separate fractions, which are distilled off as they are collected, and weighed. As soon as the weight of a single fraction drops below 5% of that of the original mixture, the elution is enhanced by the addition of the next stronger solvent in order of eluting power (*e.g.*, 100 ml petroleum ether + 10% benzene). The content of the second solvent is increased stepwise, as described above in the section on solvents, using the weights of the residues of the eluted fractions as a guide, until one reaches the second pure solvent (benzene) and so on, according to the elutotropic series, until everything has been eluted.

In order to adsorb substances which are insoluble in the chosen solvent, one can proceed in the following manner [27]. Suppose that a substance is insoluble in petroleum ether and in benzene, but soluble in chloroform. The column is prepared in benzene, 1 g of the material is dissolved in 10 ml of chloroform, and diluted with 90 ml of benzene. If a part of the solute precipitates, it is allowed to settle, and the supernatant solution is poured onto the column. The precipitate is dissolved again in 10 ml of chloroform and treated with 90 ml of benzene; the procedure is repeated until complete solution is effected. If it goes too slowly, the chloroform content is increased.

The conditions specified above can be varied widely, within certain limits (see above for dimensions of the column, ratio of adsorbed material to adsorbent, and the series of solvents). No hard and fast rules can be given for the volumes to be collected in the individual fractions, since these will depend upon the ease of separation and of elution of the particular substances involved, as well as upon other conditions. It does seem advisable, however, to relate the volumes of the fractions to the quantity of adsorbent, collecting 0.4–300 ml in each fraction for every gram of adsorbent. With columns of medium size it is best to collect 2–5 ml. With a little experience it should always be possible to separate a mixture into about 15 or more fractions, none of which contains more than 10% of the original mixture. If larger fractions do result, it is advisable to rechromatograph them.

Theoretically, there is an optimal speed with which the solvent runs through the column. If the rate is too small, undesirable diffusion effects can occur; if it is too great, the number of individual molecular adsorptions, so important for the achievement of separation, falls off. A definite rate of flow in ml/min is difficult to specify, since it depends very much upon the dimensions of the column. The rate in the absence of excess pressure can be taken as a minimum, and 2–3 times this rate, as an average (*ca.* 2–10 ml/min). This is achieved by the application of a slight pressure, which, moreover, should be kept as nearly constant as possible during the whole procedure. For an arrangement for slowing down the rate of flow *cf.* [121], and for maintaining a constant rate [123]. As a further criterion, it may be noted that a medium-scale chromatographic separation can easily be carried out in one working day; indeed, a long interruption is to be avoided.

Less demanding chromatograms for preliminary separations, or for separations

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of groups of substances, or well-worked-out routine procedures, can be performed in from one to several hours. The work can be speeded up by the use of three round-bottom flasks, which are used in turn, one for collecting the eluate, one for distillation, and one for the preliminary testing of the residue, which is immediately transferred to a small, tared boiling flask or test tube. With quantities greater than 1 g, it is often advantageous to carry out a preliminary experiment with a small sample, thus revealing possibilities for shortening the procedure, and enabling one to save time and materials in the main run. It is frequently possible to detect faintly-colored zones on the column, because of adventitious impurities; these zones should be horizontal. Hidden or inclined zones indicate irregularities in the structure of the column, which are hardly compatible with clean separations.

Further aids consist in arrangements for automatic replenishment of the solvent, and especially fraction-collectors which can be combined with automatically-recording spectrophotometers when the proper solvents are used. The changing of the solvent can also be made automatic [46]. For a method of collecting fractions with large volumes, *cf.* [122].

Instead of varying the composition of the solvent by steps, it is quite advantageous to employ the so-called gradient elution method, particularly for routine chromatography with only two different solvents [30, 393]. In this method, the eluting medium becomes continuously richer in the more polar solvent; the concentration curve can be made to be concave ($R_2 > 2\,R_1$), convex ($R_2 < 2\,R_1$) or linear ($R_2 = 2\,R_1$) (see below). For theoretical reasons a concentration curve concave above (concentration of the second solvent small at first, then rising sharply) gives the best separation, with minimum tailing. Fig. 1 illustrates the apparatus employed successfully by LAKSHMANAN AND LIEBERMAN [30], using benzene and ethanol (separation of 17-ketosteroids in urine; see also below under specific examples). The apparatus consists of a Hershberg dropping funnel and a mixing chamber with a magnetic stirrer. The funnel contains the relatively concentrated stock solution ($C_0 = 4\%$ ethanol in benzene) and the mixing chamber, benzene ($V_0 = 700$ ml). The velocity of outflow (R_1) from the dropping funnel is regulated by the position of the tungsten wire in the capillary; that from the mixing chamber (R_2) by the cock. A uniform pressure is applied at P. (For details, consult the original article [30]; R_1 may be 0.33 ml/min, R_2, 2.5 ml/min.) From the equation $C = C_0 \left[1 - \left(\frac{a}{a+bt}\right)^{\frac{1}{b}}\right]$, where $a = V_0/R_1$ and $b = 1 - (R_2/R_1)$, one can calculate for any time t the concentration C of alcohol in the mixing chamber, and hence in the outflow.

Instead of the apparatus just described, one can also employ to good advantage one like that illustrated in Fig. 2a [36]. If both flasks are alike in shape and capacity, one has $R_2 = 2\,R_1$, and hence a linear concentration curve. By altering the shape and size of one or both flasks R_2 and R_1 can be varied at will.

With small columns, the purpose is often served by simpler arrangements, such as that of HEFTMANN AND JOHNSON [47] for the determination of corticosteroids in urine (Fig. 2b), in which a dense solvent (B) is allowed to drop into a lighter one (A),

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or that of DONALDSON *et al.* [48] for the addition of a light solvent (*A*) to a dense one (*B*) (Fig. 2c).

The individual fractions, after removal of the solvent, are tested separately, by m.p., crystal form, color reactions (*cf.* test reactions in paper chromatography,

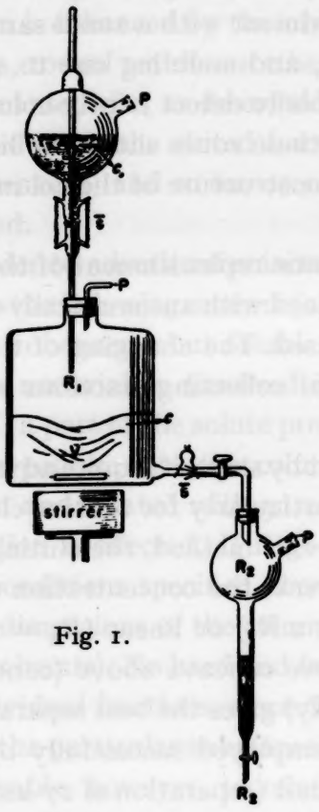

Fig. 1.

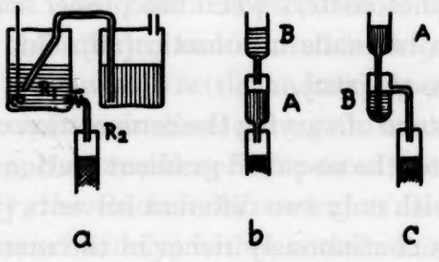

Fig. 2.

Section IV), UV- or infra-red spectra, radioactivity, or very advantageously by paper-chromatographic analysis. Fractions containing identical compounds are then combined and used for further working up, or for identification.

Another variation of the method of fractional elution consists in combining it with the so-called displacement chromatography of TISELIUS [10], in which the material is eluted not with pure solvents or mixtures thereof, but with a solution of a more strongly adsorbed material, which successively displaces the others from the adsorbent (*cf.* for example [26]). This method, however, has as yet hardly been introduced into steroid chromatography, and appears to be inferior to the classical procedure.

The method which has just been described in detail for the fractional elution from alumina is also applicable to the other common adsorbents such as magnesium silicate, silica gel, etc. It is to be observed that approximately the reverse order of eluting power of the solvents holds when charcoal is used as adsorbent, so that the adsorption of weakly polar steroids from highly polar solvents is greatest, and elution is easiest in the case of highly polar steroids and weakly polar solvents.

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7. Description of the chromatograms

The description of a chromatographic procedure should include the following data, in order to facilitate comparisons or reproduction of the work:

Source, treatment, and activity or water content of the adsorbent; ratio of the quantities of adsorbent and of material chromatographed; dimensions of the column.

Solvents used for preparation of the column, and for the chromatography; volume of eluate, and in certain cases, the volume retained by the column; velocity of filtration, pressure; if necessary, a table or curve showing the results.

8. Specific examples

(1) On *alumina*: Suitable eluting agents are petroleum ether–benzene–ether.

Several examples of the separation of isomers are given in Table 4.

One example is given in detail in Table 5 [53]: 430 mg of mixture on 20 g Al_2O_3 (Activity III), fractions of 25 ml being collected.

TABLE 4

Isomers, in order of ease of elution	*Steric position and conformation of the OH- or $OCOCH_3$-groups*	*Fusion of rings A and B*	*References*
epi-Cholesterol	3α(a)		49, 50
Cholesterol	3β(e)		
epi-Cholestanol	3α(a)	trans	49, 51
Cholestanol	3β(e)	trans	
Coprostanol (Coprosterol)	3β(a)	cis	52
epi-Coprostanol	3α(e)	cis	
7α-Hydroxycholesterol diacetate	3β, 7α(a)		53
7β-Hydroxycholesterol diacetate	3β, 7β(e)		
3β-Acetoxy-5α-cholestan-7α-ol	3β(e), 7α(a)	trans	54
3β-Acetoxy-5α-cholestan-7β-ol	3β(e), 7β(e)	trans	
Coprostanol (coprosterol)	3β(a)	cis	55
Cholestanol	3β(e)	trans	55
Cholesta-3,5-dien-7-one			56
Cholesta-4,6-dien-3-one			

TABLE 5

Fraction	*Solvent*	*7-Hydroxycholesterol diacetate*
1–6	benzene	
7–11	benzene + 5% ether	
12–13	ether	
14–25	ether	7α-isomer
26–42	ether	7β-isomer
43–48	ether + 2% methanol	

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For the separation of cholesterol and its esters *cf.* [57] for example. It is to be noted that many sterols are quite sensitive to oxidation. For the separation of calciferol and its provitamin as dinitrobenzoates, *cf.* [58].

(2) *Separations on silica gel or kieselguhr*: cholesterol palmitate-stearic acid-glycerides (petroleum ether–benzene [59]); cholestanone–cholestenone [60]; sterols as azoyl esters on silica gel-celite 2:1 [18] or silicic acid–celite 2:1 [61]; sterols as *p*-iodobenzoates with ^{131}I on silicic acid–celite 2:1 [20, 119, 124].

(3) *Aluminum silicate.* For the isolation of sterols or their glycosides directly from natural oils, *cf.* [22, 23].

(4) *Florisil*: *cf.* [184].

(*b*) *Steroids*

(1) *Alumina.* The solvents most frequently employed for elution are petroleum ether–benzene–ether–chloroform, and benzene or carbon tetrachloride–alcohol.

Table 6 presents the separations of compounds which differ only in their configuration at position 3 and/or in the joining of rings A and B.

TABLE 6

Isomers, in order of ease of elution	*Stereochemistry*		*References*
	OH or OCOR at C_3	*rings A and B*	
Androsterone	α (a)	trans	43, 62
3α-Hydroxy-5β-androstan-17-one (etiocholanolone)	α (e)	cis	
3β-Hydroxy-5β-androstan-17-one	β (a)	cis	63
Androsterone	α (a)	trans	
Androsterone acetate or -benzoate	α (a)	trans	64
3β-Hydroxy-5α-androstan-17-one acetate or -benzoate (*epi*androsterone acetate or -benzoate)	β (e)	trans	
3α-Hydroxy-5β-androstan-17-one benzoate	α (e)	cis	64
3β-Hydroxy-5β-androstan-17-one benzoate	β (a)	cis	
3β-Hydroxy-5α-androstan-17-one benzoate	β (e)	trans	64
3β-Hydroxy-5β-androstan-17-one benzoate	β (a)	cis	difficultly separable
3β-Hydroxy-5α-pregnan-20-one benzoate (*allo*pregnanolone benzoate)	β (e)	trans	64
3β-Hydroxy-5β-pregnan-20-one benzoate	β (a)	cis	difficultly separable
3α-Hydroxy-5α-pregnan-20-one	α (a)	trans	65
3α-Hydroxy-5β-pregnan-20-one (pregnanolone)	α (e)	cis	
21-Acetoxy-3β-hydroxy-5β-pregnan-11,20-dione	β (a)	cis	66
21-Acetoxy-3α-hydroxy-5β-pregnan-11,20-dione	α (e)	cis	
3α,11β,17α,21-Tetrahydroxy-5α-pregnan-20-one diacetate	α (a)	trans	67
3β,11β,17α,21-Tetrahydroxy-5α-pregnan-20-one diacetate	β (e)	trans	
5α-Androstan-3,17-dione	—	trans	65
5β-Androstan-3,17-dione	—	cis	
5α-Pregnane-3,20-dione	—	trans	65
5β-Pregnane-3,20-dione	—	cis	

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Further examples of steroids with stereoisomerism of the substituents at carbon atoms 11, 17, and 20 are found in Table 7. For the connection between structure and chromatographic behavior, see section 10, p. 127.

VON EUW AND REICHSTEIN [72] give an instructive example of the separation of natural products. In this instance 3.3 g of a purified, acetylated, ketonic fraction of weakly polar components of beef adrenals was chromatographed on 90 g of alumina. Fractions of 400 ml were collected (Table 8). (It may happen, on acylation of the mixture with acid anhydride and pyridine containing a little sulfur, that the sulfur

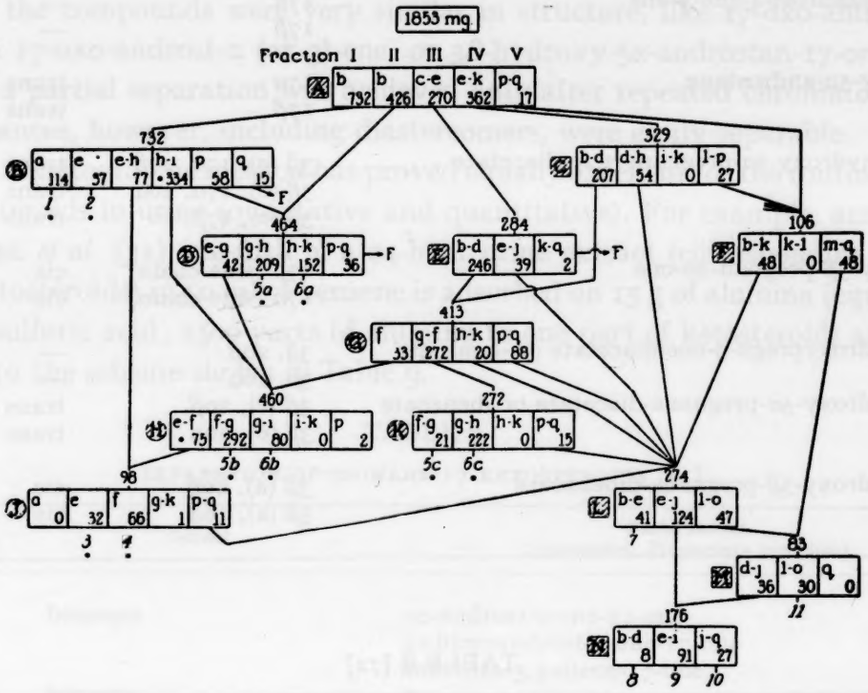

Fig. 3. Schema of a systematic chromatographic separation of α-keto steroids from urine [43]. a = petroleum ether–benzene (1:1); b = CCl_4; c = CCl_4–benzene (1:3); d = CCl_4–benzene (1:1); e = benzene; f = benzene–ether (9:1); g = benzene–ether (8.5:1.5); h = benzene–ether (4:1); i = benzene–ether (3:1); j = benzene–ether (1:1); k = ether; l = ether–methanol (49:1); m = ether–methanol (19:1); n = ether–methanol (9:1); o = ether–methanol (1:1); p = methanol; q = acetic acid. The shaded squares denote magnesium silicate as adsorbent, and the shaded circles, Al_2O_3. • = crystalline fractions. The large letters indicate the individual chromatograms. The quantities of ketosteroids are expressed in equivalents of androsterone (color reaction).

impurity enters the mixture. During chromatography, the sulfur then appears in the eluate with petroleum ether or petroleum ether–benzene.)

For further examples from the field of adrenal steroids, see [66, 73, 74].

As an example from the comprehensive studies of DOBRINER *et al.* [43, 63, 65] on the isolation of urinary steroids, the schema in Fig. 3 illustrates a systematic fractionation of the so-called α-keto steroids*.

Since some compounds were unstable on alumina, magnesium silicate was chosen as a substitute from among many adsorbents tested, because it gave the best yields from the standpoint of weight and of chromogenic value. Since the yields

* *Translator's note.* The α-keto steroids are those not precipitated by digitonin.

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TABLE 7

Isomers, in order of ease of elution	*Stereochemistry*		*References*
	substituents	*rings A and B*	
Methyl-3β,11-diacetoxy-12-oxoetianate	3β (a), 11α (e)	trans	68
	3β (e), 11β (a)	trans	
3β,17-Dihydroxyandrost-5-ene-3-acetate or -benzoate	3β, 17α	—	64
	3β, 17β	—	
17-Hydroxyandrost-4-ene-3-one	17α	—	64
	17β	—	
17-Hydroxy-5α-androstane	17α	trans	69
	17β	trans	
3,17,20-Trihydroxy-5α-pregnane-3,20-diacetate	3β (e), 17α, 20β	trans	
	3β (e), 17α, 20α	trans	70
	3β (e), 17β, 20	trans	
3α-Hydroxy-5β-pregnan-20-one	17β (side chain)	cis	71
	17α (side chain)	cis	
3β,20-Dihydroxypregn-5-ene-diacetate or -benzoate	3β, 20β	—	64
	3β, 20α	—	
3β,20-Dihydroxy-5α-pregnane-diacetate or -benzoate	3β (e), 20β	trans	64
	3β (e), 20α	trans	
3β,20-Dihydroxy-5β-pregnane-dibenzoate	3β (a), 20β	cis	64
	3β (a), 20α	cis	

TABLE 8 [72]

Fraction	*Solvent*	*Eluate*
1	70% petroleum ether + 30% benzene	
2	55% petroleum ether + 45% benzene	3β-hydroxy-5α-pregnan-20-one-acetate (*allo*pregnanolone)
3	40% petroleum ether + 60% benzene	
4	25% petroleum ether + 75% benzene	
5–6	benzene	progesterone
7	benzene	
8–10	benzene	androst-4-ene-3,17-dione crystals melting at 277–278°
11–13	benzene	
14	benzene	
15–16	99% benzene + 1% ether	crystals melting at 234–236°
17	98% benzene + 2% ether	
18	98% benzene + 2% ether	crystals melting at 242–245°
19	98% benzene + 2% ether	3β,11β-dihydroxy-5α-pregnan-20-one-acetate
20	96% benzene + 4% ether	
21	94% benzene + 6% ether	adrenosterone
22	92% benzene + 8% ether	
23	88% benzene + 12% ether	crystals melting at 259–261°
24	85% benzene + 15% ether	17β-hydroxyprogesterone
25	80% benzene + 20% ether	
26	70% benzene + 30% ether	17a-methyl-D-homo-androst-4-ene-17a-ol-3,17-dione
27	50% benzene + 50% ether	
28–36	ether and ether with 1–60% acetone	various high-melting crystals

References p. 186.

were satisfactory with weakly- and moderately polar ketosteroids, and losses occurred only with the more strongly polar fractions (eluted after 3α-hydroxy-5β-androstan-17-one (etiocholanolone)) alumina was also used along with magnesium silicate. Magnesium silicate was employed for the preliminary separation (chromatogram A, Fig. 3). For further fractionation Al_2O_3 or magnesium silicate was used, depending upon the polarity of the groups. By combining suitable eluates from various chromatograms, and rechromatographing, very complex mixtures could be separated into their components.

When the compounds were very similar in structure, like 17-oxo-androsta-3,5-diene* and 17-oxo-androst-2 (or 3)-ene, or 3β-hydroxy-5α-androstan-17-one and its Δ^5-analog, a partial separation was achieved only after repeated chromatographing. Most substances, however, including diastereomers, were easily separable.

The chromatographic method has proved equally excellent for the routine analysis of 17-ketosteroids in urine (qualitative and quantitative). For example, according to DINGEMANSE *et al.* [31] one-fifth of a 24-hour urine extract (corresponding to about 6 mg 17-ketosteroids) in 50 ml of benzene is adsorbed on 15 g of alumina (equilibrated over 58% sulfuric acid; 2500 parts of alumina to one part of ketosteroid) and eluted according to the scheme shown in Table 9.

TABLE 9

SEPARATION OF URINARY 17-KETOSTEROIDS [31]

Fraction No. (50 ml fractions)	*Solvent*	*Eluted steroids (colorimetric, Zimmerman procedure)*
1–3	benzene	5α-androst-2-ene-17-one 3-chloroandrost-5-ene-17-one androsta-3,5-diene-17-one
4–5	benzene	ø
6–8	benzene	i-6-hydroxyandrostan-17-one
9–10	benzene + 0.1% ethanol	ø
11–13	benzene + 0.1% ethanol	dehydro*epi*androsterone *epi*androsterone
14	benzene + 0.1% ethanol	ø
15–19	benzene + 0.1% ethanol	androsterone, 3α-hydroxy-5α-androst-9-ene-17-one
20–21	benzene + 0.1% ethanol	ø
22–30	benzene + 0.1% ethanol	3α-hydroxy-5β-androstan-17-one 3α-hydroxy-5β-androst-9-ene-17-one
31–32	benzene + 0.1% ethanol	ø
33–38	benzene + 0.5% ethanol	11β-hydroxy-5α-androstan-17-one + ?
39–40	benzene + 0.5% ethanol	ø
41–43	benzene + 0.5% ethanol	3α,11β-dihydroxy-5β-androstan-17-one
44	benzene + 2% ethanol	ø
45–48	benzene + 2% ethanol	various ketosteroids

If the optical densities of the various fractions are plotted against the numbers of the fractions or against the eluted volume, very uniform and characteristic curves are obtained. This method has been much used for clinical purposes, and also much modified (*cf.*, for example [40, 41, 42].) LAKSHMANAN AND LIEBERMAN [30] have worked

* *Translator's note.* The term "keto" was formerly employed to denote a carbonyl group.

References p. 186.

TABLE 10

SEPARATION OF 3,5-DINITROBENZOATES INTO GROUPS OF MONO-, DI- AND TRIHYDROXY STEROIDS [76]

	Groups	*Eluting agent*
I	Cholesterol	petroleum ether–benzene 9:1
II	3α,20α-Dihydroxy-5α-pregnane 3α,20α-Dihydroxy-5β-pregnane 3α,17β-Dihydroxy-5α-androstane 3β,17β-Dihydroxyandrost-5-ene	petroleum ether–benzene 1:1
III	3α,12α,20-Trihydroxy-5β-pregnane 3β,16α,20β-Trihydroxy-5α-pregnane	petroleum ether–benzene 1:4

out a very elegant variation, using the gradient-elution technique, which permits the complete 17-ketosteroid analysis of a urine extract within 5 hours; *cf.* [393]. The 3,5-dinitrobenzoates have also been employed for the determination of urinary steroids [75, 76]; in this case, the attempt is usually made only to separate into esters of mono-, di-, and tri-hydroxy compounds. As an example, the dinitrobenzoates of the steroids listed in Table 10 can be separated on alumina (Activity II–III) [76].

(2) *Silica gel.* The last few years have witnessed a great increase in the importance of chromatography on silica gel, particularly for the more strongly-polar steroids, whose handling on alumina is either not possible at all, or only with large losses. Silica gel is suitable not only for the separation of groups, but also for very delicate fractionations, provided that the proper conditions are selected. An example of the separation of strongly polar steroids into groups is given in Table 11 [77]. A 30–60 fold quantity of silica gel was employed.

The less-polar 17-ketosteroids have also been fractionated on 200 times their

TABLE 11

PRELIMINARY FRACTIONATION OF STRONGLY POLAR URINARY STEROIDS ON SILICA GEL [77]

Fraction	*ml*	*Solvent*	*Eluate*
1	25–35	benzene	—
2	50	benzene–ether 2:1	—
3	50	benzene–ether 1:1	Pregnanetriol
4	100	benzene–ether 1:2	
5	50	ether	
6	50	ether–ethyl acetate 9:1	3β,17α,21-trihydroxy-5α-pregnan-20-one
	50	ether–ethyl acetate 4:1	tetrahydrocortisone
	50	ether–ethyl acetate 3:1	cortisol, cortisone
7	50	ether–ethyl acetate 2:1	dihydrocortisone
	50	ether–ethyl acetate 1:1	11β,17α,21-trihydroxy-5β-pregnan-3,20-dione
	100	ether–ethyl acetate 1:2	corticosteron
8	50	ethyl acetate	tetrahydrocortisone
9	50	acetone	cortisol
10	50	methanol	cortisone, corticosterone
			dihydrocortisone

References p. 186.

TABLE 12

SEPARATION OF CORTICOSTEROIDS ON 60–100 PARTS OF SILICA GEL [78]

Eluting Agents		*Eluted Substances*
a	*b*	
benzene–ethylacetate	benzene–ether	
7:1–4:1	4:1–2:1	progesterone
4:1–3:1	2:1	pregnenolone
	2:1–1:1	17α-hydroxyprogesterone
1:1		17β-hydroxyprogesterone
1:1	ether–ethyl acetate 4:1	corticosterone
1:1–1:3	4:1–1:1	cortisol

weight of silica gel [44]; to be sure, alumina appears to give superior results in this case. However, inactivated silicic acid (water content 17%) has yielded good separations with 17-ketosteroids, too [120]. Silica gel is often employed for the separation, or separation into groups, of corticosteroids extracted from blood or organs. *Cf.* for example, the elution sequence in Table 12 [78].

For the separation of androst-4-ene-3,17-dione, 6α-,6β-, and 19-hydroxy-androst-4-ene-3,17-dione with the same series of solvents, *cf.* [79]. For the group separation of corticosteroids for microdetermination with chloroform and chloroform + 0.5–10% ethanol, *cf.* [45].

The use of chloroform–acetone mixtures without ethyl acetate or alcohol, for the separation of corticosteroids on 30–60 parts of silica gel, has worked out well (Table 13a [36]). When chloroform poor in alcohol is used, hardly any corticosteroids can be eluted, a little cortexone appearing at the most, when a large amount of solvent is used.

The tailing effect causes some difficulty in obtaining a clean separation when the

TABLE 13

SEPARATION OF PURE CORTICOSTEROIDS ON SILICA GEL WITH CHLOROFORM–ACETONE MIXTURES [36]

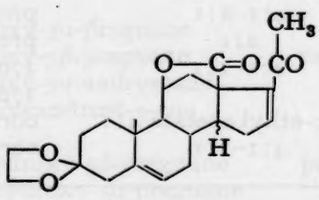
% Acetone in Chloroform	*Eluted Steroids*
	(*a*) *activated silica gel*
10–15	17α-hydroxycortexone*
10–25	corticosterone
15–30	cortisone
30–40	cortisol
	(*b*) *inactivated silica gel*
1–4	11-dehydrocorticosterone
3–6	17α-hydroxycortexone
6–10	corticosterone
10–14	cortisone
15–20	aldosterone
20–35	cortisol

* *Translator's note.* Cortexone is 21-hydroxypregn-4-ene-3,20-dione (11-deoxy-corticosterone)

References p. 186.

usual, activated silica gel is used. Considerably better separations are obtained with inactivated silica gel (water content 20%, for example) and the gradient elution technique (see Table 13b). The compounds can then be eluted with even lower acetone concentrations and the tailing is avoided to a large extent [36].

Table 14 gives a notable example of an excellent separation of stereoisomers [81].



(VI 14*a*)
(VII 14*β*)

In the total synthesis of aldosterone, the racemic intermediates VI and VII, *d,l*-11 → 18-lactones of 3-ethylenedihydroxy-11*β*-hydroxy-20-oxopregna-5,16-diene-18-oic acid, were obtained.

Twelve grams of the crystallized mixture, dissolved in benzene–ethyl acetate (9:1) were adsorbed on 1.2 kg of silica gel (Davison, through 200) in benzene–ethyl acetate (9:1). The dimensions of the column were 7.4 × 54 cm; the flow rate, under pressure, 45 ml/min. If the ethyl acetate concentration at the beginning was increased only to 8.5:1.5, no separation was possible!

(3) *Magnesium silicate.* This adsorbent is less strongly polar and less selective than alumina, but, like silica gel, it is very useful for the chromatography of strongly

TABLE 14

SEPARATION OF THE STEREOISOMERS VI AND VII ON SILICA GEL [81]

Fraction	*Vol. (l)*	*Solvent*	*Eluate (mg)*	
1–11	4 each	benzene–ethyl acetate 9:1	ø	
12	4	benzene–ethyl acetate 9:1	3, oil	
13–14	4 each	benzene–ethyl acetate 9:1	ø	
15	4	benzene–ethyl acetate 9:1	287	crystalline VI (14*a*)
16	4	benzene–ethyl acetate 9:1	574	
17	20	benzene–ethyl acetate 9:1	4325	
18	20	benzene–ethyl acetate 9:1	2294	
19	20	benzene–ethyl acetate 9:1	1146	
20	20	benzene–ethyl acetate 9:1	570	
21	8	benzene–ethyl acetate 9:1	103	
22	4	benzene–ethyl acetate 9:1	35	
23	4	benzene–ethyl acetate 9:1	28	
24	2	ethyl acetate	120	
25	4	ethyl acetate	804	crystalline VII (14*β*)
26	4	ethyl acetate	362	

polar compounds, which are very difficult to elute from alumina. Examples have already been given of the separation of urinary steroids [43] (under *Alumina*, p. 116 ff). It is also much employed for the strongly polar corticosteroids, as, for example, for concentrating them out of extracts of plasma with chloroform + 2–25% of

methanol or 2–15% of ethanol [34], or for isolating cortisone from urine with benzene–ether–ethyl acetate mixtures [93], or for isolating corticosteroids from adrenals with petroleum ether–methylene chloride–acetone–ethanol or petroleum ether–ethyl acetate [46]. For further examples of the use of florisil, *cf.* [185–188].

(4) *Charcoal.* This adsorbent is little used for steroids, since it is not entirely safe [39]. It has been employed either for purposes of purification [25] or, admixed with celite, for the separation of isomers [38]. The ratio of charcoal to celite is 1:2 in methanol; elution of derivatives of 5β-pregnanes is carried out with acetone, and of 5α-pregnane derivatives, with methylene chloride.

(*c*) *Estrogens*

Adsorption chromatography on alumina has thus far been used only relatively seldom for the free compounds. *Cf.* [83] for a rough fractionation of urinary estrogens into estrone, estradiol, and estriol fractions; for the separation of the methyl ethers of estrone and estradiol on alumina (Activity II) with petroleum ether–benzene 6:4, *cf.* [84]. More delicate separations could be achieved [128] on florisil, with benzene–acetone and the gradient elution technique. A satisfactory separation of estriol, estradiol and estrone, in that order, by non-ionic adsorption on Amberlite IRC-50 has recently been described [390].

(*d*) *Glycosides and aglycones (cardenolides and bufadienolides)*

REICHSTEIN and his co-workers in particular have carried out numerous investigations in this field. They usually used benzene–chloroform–methanol and alkali-free alum-

TABLE 15

CHROMATOGRAPHY OF GLYCOSIDES ON Al_2O_3 [87]

Fraction No. (200ml each)	*Solvent*	*Quantity in (mg)*	*Eluate, tested by paper chromatography*
1–3	benzene–chloroform 8:2	164.9	amorphous
4–5	benzene–chloroform 6:4	80.0	glycosides A, B, C
6–10	benzene–chloroform 1:3	743.9	A, B, C, D
11–12	benzene–chloroform 1:3	27.1	A, B, C, D, F
13–14	benzene–chloroform 1:3	3.5	
15–16	chloroform	12.2	C, D, F
17	chloroform–methanol 99:1	308.6	C, D, E, F
18	chloroform–methanol 99:1	149.1	D, E, F
19–21	chloroform–methanol 99:1	60.4	D, E, F
22	chloroform–methanol 99:1	23.0	D, E, F, G
23–25	chloroform–methanol 98:2	268.5	F, G
26–28	chloroform–methanol 98:2	32.1	G
29–31	chloroform–methanol 96:4	32.7	G, (H?)
32–33	chloroform–methanol 92:8	12.1	positive
34	chloroform–methanol 85:15	12.1	negative
35	chloroform–methanol 70:30		
36	chloroform–methanol 40:60		

C = Vanderoside E = Kwangoside G = Sarnovide
D = Sarmentocymarin F = Emicymarin H = Odoroside

References p. 186.

ina or magnesium silicate. The latter adsorbent is more gentle than alumina, which can easily cleave acetic acid out of 16-acetoxy-compounds [85, 86]. Adsorption chromatography is often employed for preliminary purification, or for separation into groups, partition chromatography (*q.v.*) being employed subsequently. An example of the preliminary separation of 2.2 g of ethereal extract of *Strophanthus* seeds on 64 g of alkali-free Al_2O_3 is given in Table 15 [87].

Further examples of the use of alumina or magnesium silicate–celite are given [32, 85, 86, 88–92, 94, 95] among others. Similarly, toad poisons (bufotalin, marinobufagin, bufotalinin, telocinobufagin) can be separated in a preliminary manner on alumina [96, 97]. For chromatography on silica gel, *cf.* [129].

(*e*) *Sapogenins*

WALENS *et al.* [98] report a group separation of mono- and dihydroxysapogenins on alumina or magnesium silicate (florisil). The substances are eluted from florisil with (1) benzene, (2) benzene + 5% chloroform, (3) benzene + 20% chloroform, (4) benzene + 20% ethanol. Fractions (1) and (2) contain the monohydroxysapogenins (hecogenin, diosgenin, tigogenin, sarsasapogenin, and similagenin) while fractions (3) and (4) contain the dihydroxysapogenins (kammogenin, chlorogenin, manogenin, gitogenin, markogenin, samogenin, yuccagenin, kryptogenin, rockogenin).

From alumina the materials are eluted with (1) benzene + 5% chloroform, (2) benzene + 20% chloroform, (3) chloroform, (4) benzene + 20% ethanol. The monohydroxysapogenins are again found in fractions (1) and (2), and the dihydroxy compounds in fractions (3) and (4). *Cf.* also [189].

(*f*) *Bile acids*

The bile acids in the form of methyl esters are excellently suited to chromatography on alumina. An example [99] is the separation of the methyl esters of 3α-acetoxycholl-11-enic acid (VIII) and its 3β-isomer (IX) presented in Table 16. Approximately

TABLE 16

CHROMATOGRAPHY OF THE ISOMERIC BILE ACID METHYL ESTERS VIII AND IX ON ALUMINA [99]

Fractions	*Solvent*	*Eluate*
1–3	Petroleum ether	
4–11	Petroleum ether–benzene 9:1	VIII
12–13	Petroleum ether–benzene 7:3	VIII
14–15	Petroleum ether–benzene 1:1	VIII
16–22	Petroleum ether–benzene 1:4	IX
23–24	Benzene	IX
25–28	Benzene–ether 9:1	IX
29	Benzene–ether 8:2	traces
30	Benzene–ether 1:1	
31	Ether	
32	Methanol	

References p. 186.

300 mg of a crude mixture were separated into fractions of 22 ml with the aid of 6.5 g of alumina (standardized according to BROCKMANN).

In the same work, the separation of the methyl esters of 11,12-dibromo-, 11α,12α-oxido- and 12-oxochol-9-enoic acids is described. Another interesting example is the separation of methyl esters of 11-cholenic, 11-oxocholanic, and 12-oxochol-9(11)-enic acids with the same series of solvents as above [100]. For the separation of the 9,11- and 11,12-oxido derivatives of 3α-acetoxycholanic acid methyl ester, *cf.* [101]; for separation of bile acids as azoyl amides on alumina [19], or as azoyl methyl esters on magnesium carbonate [21].

(g) *Conjugated steroids*

Sulfates and glucuronides of urinary steroids have recently been chromatographed on alumina with butanol–water [126] or with ethanol–water or phosphate–citrate buffer [127].

9. Side reactions of steroids with the adsorbents

At the beginning of this chapter it was stated that one pre-requisite for successful adsorption chromatography was that the adsorbed material must not be altered by the adsorbent. Because of the large choice of adsorbents which may be employed this condition can almost always be met. Nevertheless, a few side-reactions of alkaline alumina with certain steroids must be pointed out here; they demands special precautions [27].

(1) 17-Hydroxy-20-ketones, especially those of the 17α-pregnane series can be rearranged to D-homoketones (X and XI). This can be prevented to a large extent by the use of alkali-free alumina of lower activity, and with the 17-acetates, it can be avoided altogether (*cf.* also [104]).

(2) Δ^5-3-Ketones are rearranged to Δ^4-3-ketones with alkaline alumina, but not with alkali-free [27] (XII and XIII).

(3) Free hydroxyketones (α-ketols, XIV) are partly decomposed even by alkali-free alumina; it is best, therefore, to use the corresponding acetates.

(4) 14β-21-Oxido-23 → 21 lactones (XV) of cardenolides (digitalis, strophantus) suffer great losses with all sorts of alumina, presumably by splitting of the lactone ring.

(5) The oxo-21 → 14 lactones of the 14β-series (XVI) behave likewise.

(6) The splitting of acetic acid from certain acetoxy compounds, *e.g.* the cardenolides (XVII, XVIII) [86]. This reaction does not occur with magnesium silicate as adsorbent. The Δ^7 compound is easily formed from 7α-hydroxy-cortexon diacetate.

(7) 11,12-Bromohydrins split HBr to give epoxides (XIX, XX [99]) on alkaline alumina, but not on neutral alumina.

(8) The bromine of bromo-compounds can be replaced by chlorine (XXI, XXII) with alumina which has been washed until neutral with dilute hydrochloric acid.

(9) Hydration of epoxides (XXIII, XXIV [107]).

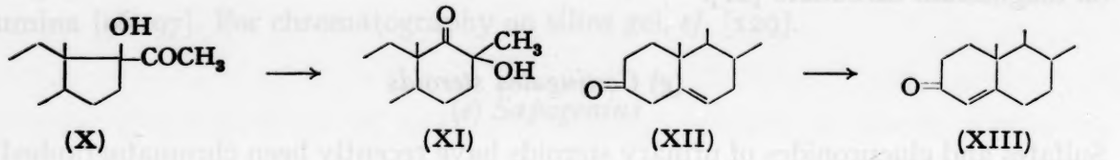
References p. 186.

(10) Dehydration of steroid cyanhydrins, depending upon the kind of aluminum hydroxide employed (XXV–XXVII [108]).

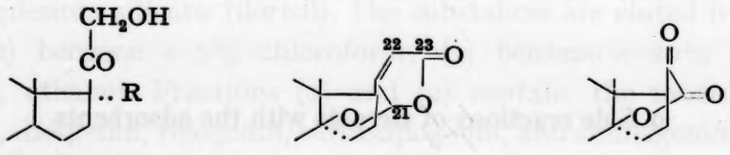
(11) Replacement of bromine by OH in allyl compounds (XXVIII, XXIX [109, 110]).

(12) Saponification of steroid benzoates [111] and acetyl migration [112] have also been observed. Cholesterol-ozonide could not be eluted again after being adsorbed on alumina [113].

All these side reactions become more marked the longer the compounds remain

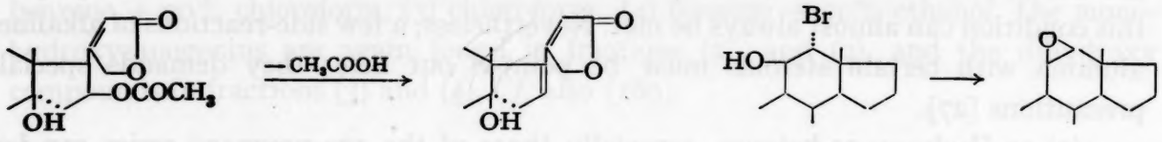


(X) (XI) (XII) (XIII)

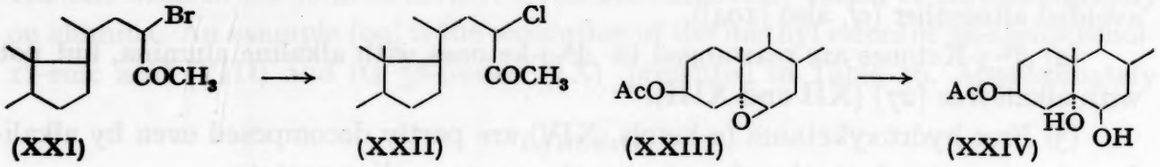


R=H, OH

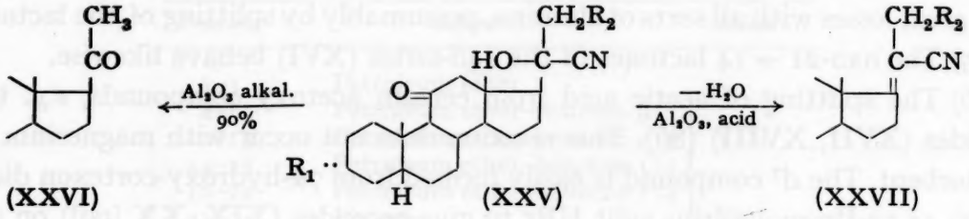
(XIV) (XV) (XVI)



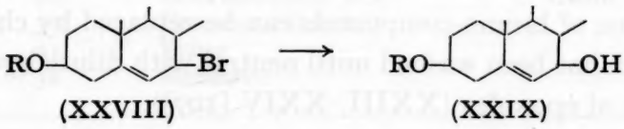
(XVII) (XVIII) (XIX) (XX)



(XXI) (XXII) (XXIII) (XXIV)



(XXVI) (XXV) (XXVII)



(XXVIII) (XXIX)

References p. 186.

in contact with alumina. They can often be eliminated to a large extent by working rapidly.

Such side reactions have hardly been proved to occur at all on more weakly active adsorbents like *silica gel* or *magnesium silicate* (*cf.*, however [114] for the isomerization of sterols on silica gel in halogen-containing solvents). MATTOX AND MASON [115] have recently reported the destruction of α-ketols on silica gel (in combination with formamide); in several other laboratories this did not occur, however. Losses or alterations in steroids have occasionally been observed with completely anhydrous silicic acid [120].

Charcoal can lead to partial decomposition of steroids, especially those with dioxyacetone side chains; thus, for example, cortisone yields adrenosterone [39]. The various types of compounds and conditions involved have not yet been sufficiently investigated to make possible any general statements.

10. Constitution and chromatographic behavior (elution series)

A sequence of groups which are increasingly adsorbed, and eluted with decreasing ease, has already been given in Section 3 A 2, page 105. To supplement these data, various generalizations can be made [63, 65]; they are set out in Table 17 (*cf.* also under specific examples, Tables 4–7).

TABLE 17

ORDER OF ELUTION OF CORRESPONDING DERIVATIVES OF STEROIDS [63]

Alumina columns
1. Monoketones
2. Monoketones with 1 acetoxy group
3. Diketones
(a) 5α-pregnanes
(b) 5β-pregnanes
(c) 5α-androstanes
(d) 5β-androstanes
4. Monohydroxy-monoketones
(a) 5α-pregnanes
(b) 5β-pregnanes
(c) 5α-androstanes
(d) 5β-androstanes
Magnesium silicate columns
1. C_{19}-Dihydroxy monoketones
2. C_{19}-Monohydroxy diketones
3. C_{21}-Dihydroxy monoketones

The following points are to be noted in this connection:

(1) C_{21}-Pregnane derivatives are eluted before the corresponding C_{19}-androstane derivatives.

(2) 3-Hydroxysteroids with rings A and B *trans* to one another (5α-androstanes

References p. 186.

and 5α-pregnanes) are eluted before the corresponding isomers with rings A and B in the *cis* position (5β-androstanes and 5β-pregnanes) provided that the 3-hydroxyl groups in the compounds compared are both either α or β. If they are not, as in the case of 3β-hydroxy-5β-androstan-17-one and 3α-hydroxy-5α-androstan-17-one, the rule does not hold, or may not hold; in this particular case, for example, the 5β-derivatives are eluted before the 5-α (*cf.* Table 4). This is understandable, if the chromatographic behavior is determined not only by the method of fusion of the rings A and B, but also to at least an equal extent by the steric position of the hydroxyl groups at C_3.

(3) Saturated compounds are eluted before their unsaturated analogs, except for example in the case of hydroxy-5β-androstanone, which is eluted after hydroxy-5α-androst-9(11)-enone. On the other hand, 5α-androst-1-en-3,17-dione is eluted, as expected, after 5α-androstan-3,17-dione. In the elution series, the 5α-androst-1-enedione comes before the androst-4-enedione, however. The separation of saturated and Δ^5 unsaturated steroids is sometimes quite difficult. RUZICKA AND PRELOG [116] avoided this difficulty by converting the Δ^5- compound into a 5α,6α-diol, which was easily separable from the unchanged, saturated compound (*cf.* also [64[).

(4) The diketomonohydroxy compounds are eluted before the dihydroxymonoketones, with the significant exception that the dihydroxy compounds with an OH-group at C_{11} are eluted before the diketo-compounds with a keto-group at C_{11}; for example 11β-hydroxy-5α-androsterone (3α,11β-dihydroxy-5α-androstan-17-one) and 3α,11β-dihydroxy-5β-androstan-17-one come off first, and 11-ketoandrosterone (3α-hydroxy-5α-androstan-11,17-dione) and 3α-hydroxy-5β-androstan-11,17-dione only later.

In its general features, the elution series observed with alumina holds also for other hydrophilic adsorbents. Where small differences are involved, the rules can not, however, be transferred without reservation. Table 18 presents some information on this point.

TABLE 18

Order of elution (→) on	
alumina	*magnesium silicate*
↓ 3α-hydroxy-5α-androstan-11,17-dione	↑
↓ 3α-hydroxy-5β-androstan-11,17-dione	↑
↓ 3β-hydroxy-5α-androstan-17-one	↑
↓ 3β-hydroxy-androst-5-en-17-one	↑
↓ 3α,6α-dihydroxy-5α-pregnan-20-one	↑
↓ 3α,6β-dihydroxy-5β-pregnan-20-one	↑

These observations about constitution and chromatographic behavior also hold in general for acetates and benzoates of the compounds mentioned.

Epimeric hydroxysteroids ([64, 65, 63] and Tables 4–7).

As far as the 3-hydroxysteroids are concerned, the 3α-compounds are usually

eluted before the 3β-, if the only difference is isomerism at C_3 [51, 64, 67, 117]. For exceptions, see [66, 63, 52]. Among hydroxy compounds, 17α-derivatives are always eluted before 17β-derivatives [64, 69, 70], whether the other valence at C_{17} is occupied by a hydrogen atom or a carbon side chain. The 20β-isomers of 20-hydroxy compounds are eluted before the 20α-isomers [64, 70], the benzoates being easier to separate than the acetates or the free compounds [64].

Many comparative studies on the order of elution of adsorbed steroids remain to be done, so that it is for the present hardly possible to present general rules covering the mutal influence upon the chromatographic behavior of isomerism at, say, C_{11} and C_3, or C_{11} and C_5. In contrast to partition chromatography, where there is good correlation between the rate of migration of a compound and the conformation (axial or equatorial) of its hydroxyl groups, such is not the case in adsorption chromatography. Sometimes compounds with axial groups, and sometimes those with equatorial groups are eluted first. BARTON (quoted in [64]) has pointed out that this irregularity may be attributable to the fact that substances adsorbed on a rigid surface might possibly possess different conformations from those in solution.

B. PARTITION CHROMATOGRAPHY

1. Methods of procedure

MARTIN AND SYNGE [12] introduced the method of partition chromatography, which is based on the same principles as the counter-current distribution method of CRAIG. The principle of the method of fractional elution is employed, as described above in the section on adsorption chromatography (3 A 1, page 104). The possibilities for identifying the colorless steroids in the eluate have also been mentioned there. An earlier section (2) contains a discussion of the advantages and disadvantages of adsorption and partition chromatography, and mentioned several criteria which are decisive in choosing the one method or the other.

Without going into the theory of partition methods, we merely recall that the distribution coefficient of a substance is independent of its concentration within wider limits than is the adsorption coefficient. At higher concentrations, however, the distribution coefficient usually decreases, leading to a broadening of the bands. Similar limitations are imposed by irregular flow through the column or accidental displacement effects. Furthermore, a pure partition procedure is a relatively rare occurrence; depending upon the particular system involved, greater or smaller adsorption effects will occur concomitantly. This can interfere, by causing tailing, for example, or it can even be advantageous, since the combined action may make possible a better separation.

A particular advantage of partition chromatography consists in the fact that the solvent systems can be varied over a wide range, and hence adapted to the most varied requirements. One normally works with two phases of limited miscibility, and a hydrophilic supporting medium which is as inert as possible. The supporting medium

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binds the more strongly polar phase, rendering it stationary; the less polar phase is mobile, and passes from top to bottom. It is sometimes even possible to constitute stationary and mobile phases out of two completely miscible solvents, if one is preferentially bound to the solid support.

In counter-current distribution, the best results are achieved by selecting phases such that at equal volumes the average distribution coefficient of the sample is approximately 1. In column chromatography, the relative volumes of the phases are fixed within narrow limits, the ratio of the volume of the mobile phase to that of the stationary phase being not 1, but usually 5–10; hence the solvent system must be so chosen that the distribution coefficient $\left[\frac{\text{concentration in mobile (non-polar) phase}}{\text{concentration in stationary (polar) phase}}\right]$ lies between 0.2 and 0.1. Another possibility is to make the supporting medium hydrophobic, whereupon the non-polar solvent becomes the stationary phase, and the polar solvent, the mobile phase. This procedure, with reversed phases, due to HOWARD AND MARTIN [130], is particularly useful if no common solvent system can be found for a particular substance which gives a distribution coefficient of approximately 0.1.

2. Supporting media

Neutral, porous, fine-grained materials, which adsorb only slightly or not at all are suitable as supporting media for the stationary phase. *Kieselguhr, silica gel* (silicic acid) and cellulose have proved especially suitable; various silicates (diatomaceous earth [329], florisil [332] are also employed occasionally. These substances can bind up to 100% of their dry weight of the more polar phase. The free volume of the mobile phase, on the other hand, should amount, after the column has been packed, to approximately 250–350 ml per 100 g of carrier-plus-stationary-phase. The particle size should be 100–300 mesh, in order to permit the fluid to run freely, but without the formation of channels (*cf.* Section 3 A 2, p. 105, on adsorbents). In reversed-phase chromatography, it is well to use kieselguhr which has been made hydrophobic (*e.g.* with dimethyldichlorosilane) or in some cases hydrophobic or acetylated cellulose, or rubber powder.

Kieselguhr. Good-quality products with various particle sizes can be obtained commercially, *e.g.* Hyflo Supercel, Celite 535 or 545 (Johns Manville, New York). Before use, it is cleaned by washing, for example with concentrated hydrochloric acid for 1 hour at 100°, followed by filtering, washing with distilled water until neutral, and free of ions, and drying at 110° [133, 134]. According to REICHSTEIN *et al.* [135] one washes it with a large quantity of hot distilled water until it is neutral, and extracts several times with hot methanol, benzene and chloroform in turn, until the filtrates no longer give weighable residues on evaporation. The material is finally washed again with methanol and dried for 2 days at 100°.

Silica gel, e.g., from Davison Chem. Corp., Baltimore, through 200, No. 923 or 922, can be purified by washing with ether or chloroform [47, 136, 137, 138] and used after drying.

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Cellulose is a commercial product of, for example Schleicher and Schüll or Whatman (standard grade or commercial grade). Cotton linters [131] are less suitable, for mechanical reasons. Thorough washing is particularly necessary in the case of cellulose, to prevent the occurrence of blank values. One extracts with various hot solvents, such as chloroform, ethanol, water, acetone and butanol, until the filtrates give no further residue [139, 140] and dries *in vacuo*.

Rubber powder (*e.g.*, Mealorub of Andresen Corp., Chicago or of Rubber-Stichting, Delft) is cleaned by a 24-hour extraction with acetone, dried in air, ground finely, and sieved [141].

Hydrophobic kieselguhr. Hyflo Supercel is exposed in a desiccator to the vapors of dimethyldichlorosilane, or of a low-boiling mixture of chlorosilanes, then washed, with methanol until free of acid, and dried at 100–110° [130, 142].

3. Solvents

The solvents must be as pure as possible, and, if necessary, washed until neutral (*cf.* Section 3 A 3, p. 108) if good and reproducible chromatograms are to be obtained. Obviously, the solvents must not react with the materials being chromatographed, and they must be relatively volatile. If the eluates are to be examined spectrophotometrically, the solvents must transmit in the spectral region concerned. For preparative purposes, it is particularly important that the substrate has a high absolute solubility in both phases; if it does not, only little material can be put on the column, or tailing will easily occur. Solvent systems with more than two or three components are generally very sensitive to temperature changes. Finally, the choice of the phases is determined by the distribution coefficient as mentioned above. The following solvents, among others, have been used for the substances of interest here:

(a) stationary (polar) phase:	(b) mobile (non-polar) phase:
Water	Petroleum ether
Methanol–water 1:1–1:19	Hexane
Methanol	Cyclohexane
Ethanol–water 1:3	Iso-octane
Ethanol	Benzene
Ethylene glycol	Toluene
Propylene glycol	Ether
Formamide	Isopropyl ether
Formamide–water 1:1–4:1	Methylene chloride
Nitromethane	Chloroform
Acetic acid–water 7:3	Ethylene chloride
0.4–3.1 *N* Sodium hydroxide	Ethyl acetate
0.1 *N* Sodium acetate	Ethanol (only up to 5%, in mixture with other solvents)
Citrate buffer, pH 4.0–4.5	Butanol
	Amyl alcohol
	Acetic acid (only up to 5%, in mixture with other solvents, or mixtures thereof)

When the phases are reversed the terms stationary and mobile are simply exchanged. A further selection of solvents can be made intelligently on the basis of

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experience with the Craig procedure. *Cf.* in this connection VON METSCH's compilation [143] of over 400 pairs of solvents of limited miscibility. It is perhaps superfluous to emphasize that each phase must be saturated with the other at the temperature of the experiment.

4. Equipment

Tubes used for adsorption chromatography (*cf.* Section 3 A 4, p. 110) can be employed without alteration for partition chromatography, too. The optimal length and diameter of the column are determined by the particular problem presented by the separation. The length of a column represents a certain number of theoretical separation stages, the length per stage depending in turn on factors relating to diffusion and rate of flow through the column. If the components of a mixture differ only slightly in their distribution coefficients, the column must be long in order to achieve a sufficient number of stages, and vice-versa. A 1 meter column, for example, is equal in separating power to several hundred stages in the Craig apparatus.

The cross-section of the column, on the other hand, determines the capacity of the chromatographic procedure; for example, a diameter of 2.5 cm suffices to separate approximately 200–800 mg of material, and a diameter of 10 cm, about 3–12 g. For difficultly-separable mixtures of glycosides, REICHSTEIN *et al.* [135] give, for example, the following dimensions for various weights of material (Table 19).

TABLE 19

DIMENSIONS OF PARTITION COLUMNS IN RELATION TO THE AMOUNT OF SUBSTANCE [135]

No.	*Bore*	*Useful length of the tube*	*Length of the kieselguhr-column*	*Kieselguhr–water (1:1)*	*Maximum amount of substance**	*Maximal flow-rate**
	mm	*cm*	*cm*	*g*	*g*	*ml/min*
1	26	61	50	200	0.4	0.07
2	45	90	65	700	1.4	0.3
3	55	120	90	1500	3.0	0.6
4	65	140	115	3000	6.0	1.2

* When the mixture is easy to separate (usually the case with mother-liquors and crude extracts) more material can be loaded onto the column, and the speed of flow through the column can also be increased considerably.

The top of the tube is connected by means of a ground-glass joint to an apparatus for replenishing the solvent, usually a spherical storage flask with connections for filling and, in some cases, for the application of pressure (*cf.* for example [144]). When the gradient elution technique is employed, the types of apparatus described above in Section 3 A 6, p. 111 (*cf.* also [145] for microcolumns) are useful. Even complicated separations can be made fully automatic by the use of fraction collectors (*e.g.*, the "mechanical chemist" [160]). In the partition chromatography of large amounts of material, arrangements with three columns, one above the other, have been used [146].

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5. Preparation of the column

(a) The ratio of the sample to the dry supporting medium varies within wide limits, from about 1:200 to 1:10,000. For separations of average difficulty, 500–1000 parts of supporting medium are usually sufficient.

(b) The ratio of support to the stationary phases varies in practice, for all usable solvents, within the narrow limits 1:0.5 to 1:1. That is, the supporting medium is covered with 50–100% of its own weight of the stationary phase.

(c) As far as the dimensions of the column are concerned, one should be guided essentially by the discussion in Section 4, p. 132. If the column is properly packed, the supporting medium with its bound stationary phase usually has a ratio of diameter to height ranging from 1:10 to 1:20, or occasionally 1:40.

(d) *Packing the column.* Crucial to the excellence of the separation is the proper packing of the support, charged with the stationary phase, into the tube to form a completely uniform column. It is of primary importance that the two solvents be mutually saturated (by equilibration) at the working temperature, which should be as constant as possible. The supporting medium is charged either by treating a suspension in the mobile phase with the proper quantity of stationary phase and stirring vigorously, or by mixing the supporting medium thoroughly with the stationary phase, and then suspending the material, which still appears to be "dry" in the mobile phase. In any case, one must be careful to obtain a very uniform suspension. If lumps appear, too much stationary phase has been used.

A suitable tube, with a sintered glass plate, and perhaps with a layer of sand at the bottom, is half-filled with the mobile phase. The suspension is poured in, and the stopcock at the bottom opened slightly. The slurry is allowed to settle as uniformly as possible, with frequent and thorough stirring, and successive squeezing out with a perforated plate of stainless steel attached to a rod "(Martin packer"). As soon as a solid layer has formed at the bottom, the cock is opened further and the remaining suspension squeezed out evenly. During this operation the mass must always be covered with the mobile phase, and all air bubbles should escape. When all of the column has been squeezed out, more of the mobile phase is allowed to pass through under pressure, until the material no longer settles, and a uniform, dense column is formed. The entire filling procedure is aided by tapping the column with an elastic object. With larger columns, especially, it is often practicable to carry out the packing in steps, without the individual layers being detectable later.

The finished column is covered with a protective layer of glass beads or sand, and can then be tested with a suitable dye for homogeneity and hold-up volume. A small volume (0.1–1 ml) of a concentrated solution of dye in the mobile phase is added at the top of the column, which is barely covered with solvent, and allowed to soak in slowly. Suitable dyes are those which are hardly taken up by the stationary phase, *e.g.*, fat-red, Sudan Red III, etc., and which therefore pass through the column just as rapidly as the mobile phase which is now added. The volume collected before the appearance of dye in the eluate is equal to the free volume of the mobile phase in the

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column (retention volume, threshold volume, holdup-volume, dead space, etc.). In well-packed columns the zone of dye travels evenly down the column, with a symmetrical profile in the direction of the axis, free of tongues and other irregularities. If it does not, good results can not be expected, especially when delicate separations are involved.

(e) The flow-rate, which can be regulated by the application of pressure, depends upon the cross-section of the column and the ease of separation. For a column diameter of 5–10 mm it should not exceed about 0.02–0.2 ml/min; for 10–25 mm diameter, 0.2–3.0 ml/min (*cf.* also Table 19).

Before using the column it is advantageous to wash it out thoroughly with mobile phase until no more elutable impurities can be detected (*cf.* [135, 139]).

6. Execution of partition chromatography

(a) Application of the sample to the column. The simplest procedure is to dissolve the material to be separated in the minimum possible volume (0.5–5 ml) of the mobile phase, apply this solution to the top of the column, as described above for dyes, and then rinse in with mobile phase. The solubility in the mobile phase is often too small, however; in this case one can proceed in either of two ways. One can place a layer of pure supporting medium on top of the column and apply evenly a concentrated solution of the mixture in a little of the stationary phase (0.5–1 parts, compared to the uppermost layer of pure support) and then apply the mobile phase. Alternatively, one may first impregnate a small quantity of pure supporting medium with the substance dissolved in a suitable solvent, evaporate off the solvent as completely as possible, mix the residue well with 0.5–1 part of stationary phase, and press the mixture down carefully on the top of the column; the whole procedure is repeated one or two times with smaller quantities of support, in order to obtain a quantitative transfer (*cf. e.g.* [135, 139]).

These methods for introducing the substance can be altered in various ways (*e.g.* [147]), but care must always be taken to see that the volumes of solvents employed are as small as possible, and that the ratio of stationary phase to support is the same as that of the column as originally prepared. Discs of filter paper impregnated with the substance can also be used in certain cases.

(b) The eluate is collected in fractions of size commensurate with the rate of flow (0.1–1000 ml; *cf.* the preceding section) and examined by the methods described under adsorption chromatography (3 A 6, p. 111), *e.g.* by weighing or by paper chromatography (*vide infra*). Recoveries are usually determined in order to insure quantitative elution. The elution profiles of the separated substances should be as symmetrical as possible, with only a slight asymmetry of the following edge. Long tails are a sign of poorly-packed columns, overloading, precipitation in the column, adsorption, etc.

If a separation is unsatisfactory at a given temperature, merely raising the temperature by 5–10° often produces a considerably improved result.

Under some circumstances it is possible to use a column over and over again, although the preparation of a new column often requires less time than washing the

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old one completely clean. When the technique has been standardized exactly, the elution volume of a given substance is constant and characteristic, much like the R_F-value of a paper chromatogram, and can well be used for identification.

The performance of reversed-phase chromatography is in principle identical with that just described.

7. Description of the chromatograms

In order to make possible the comparison or reproduction of the chromatograms, it is advisable to report the following data: source and treatment of the supporting medium, ratio of support to material chromatographed, extent of treatment with the stationary phase, dimensions of the column, rate of flow and pressure, composition of the phases and temperature, method of application of material to the column, retention volume of the column, volume of the fractions, and a representation of the chromatogram in the form of a table or curve.

8. Specific examples

For the separation of a mixture of products formed by the air-oxidation of cholesterol, MOSBACH *et al.* [148] used 95% aqueous methanol or propylene glycol on Celite 545 as the stationary phase and cyclohexane or petroleum ether as the mobile phase. A column (propylene glycol/petroleum ether) 1 × 30 cm with a flow rate of 0.5 ml/min yielded, for example

Cholesterol from 12.5–26 ml of eluates,
7-Ketocholesterol from 30–56 ml,
7β-Hydroxycholesterol from 125–195 ml
7α-Hydroxycholesterol from 195–225 ml.

(*b*) *Steroids*

Table 20 gives a survey of the solvent systems used for partition chromatography of various steroids; quantities ranging from a few micrograms to many grams have been separated. The method is particularly suitable for use with the relatively sensitive corticosteroids, when they are chromatographed in the unesterified form, although good results can also be obtained with adsorption chromatography by using weak adsorbents.

Many partition-chromatographic procedures are derived from that of BUTT, MORRIS AND MORRIS [133], involving the use of kieselguhr (Hyflo Supercel, Celite) as supporting medium, of aqueous alcohol as the stationary phase and hexane–benzene–chloroform–ethyl acetate as mobile phase. Systems with water, formamide or alcohol and glycols as the stationary phase supported on cellulose, kieselguhr or silica gel have been used to good effect for the isolation of aldosterone. For a detailed description of such columns *cf.* SIMPSON *et al.* [139]. Propylene glycol appears less well adapted to cellulose columns.

Automation of the separations is most advantageous for routine investigations [46, 149]. The more weakly polar corticosteroid acetates and the 17-ketosteroids can

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TABLE 20

PARTITION CHROMATOGRAPHY OF STEROIDS (ON COLUMNS)

Separation of	*Stationary phase*	*Mobile phase*	*Support*	*Remarks and references*
Androst-4-en-3,17-dione Testosterone Progesterone Cortexone Pregnanetriol(3α,17α,20α)	(7:3) methanol–water (7.5:2.5)	*n*-hexane	Hyflo Supercel or Celite 535	BUTT, MORRIS AND MORRIS [133] TAILOR [152] COX AND MARRIAN [153]
Corticosteroids	ethanol–water (2.5:7.5)	toluene	Hyflo Supercel	Separation incomplete MORRIS AND WILLIAMS [151, 154]
11-Dehydrocorticosterone + corticosterone	ethylene glycol	toluene–petroleum ether (8:2)	Hyflo Supercel	MORRIS AND WILLIAMS [154]
Corticosteroids	ethylene glycol	cyclohexane, cyclohexane–CH_2Cl_2 (10:1, 6:1, 4:1, 3:1, 2:3) and CH_2Cl_2	SiO_2	Completely automatic! Compound S (17-hydroxy-11-deoxycorticosterone) and corticosterone not separated HAINES [46, 149]
Corticosteroids, especially aldosterone	methanol–water (1:1)	benzene–petroleum ether (5:1)	kieselguhr (Celite 545)	SIMPSON AND TAIT [155, 156]
	methanol–water (1:1)	benzene–ethyl acetate (10:1) and (5:1)	kieselguhr	
	water	petroleum ether–benzene–chloroform mixtures	kieselguhr	SIMPSON *et al.* [139]
	methanol–water (3:2)	toluene–petroleum ether (67:33 to 75:25)	cellulose	
	methanol–water (1:1)	benzene or benzene–ethyl acetate (9:1)	Hyflo Supercel	HARMAN *et al.* [147]
Corticosteroids	water	petroleum ether–CH_2Cl_2 (gradient elution)	SiO_2	HEFTMANN AND JOHNSON [47, 157]
	water	benzene–ether	SiO_2	COOK *et al.* [137]
	propylene glycol	toluene	Solka Flok (cellulose)	BAKER *et al.* [158]
	propylene glycol	toluene	cellulose	separation not sharp DIRSCHERL *et al.* [140]
	formamide	benzene–$CHCl_3$ (1:1 and $CHCl_3$)	cellulose	MATTOX AND MASON [115]

	DIRSCHERL *et al.* [140]			
	formamide	benzene–$CHCl_3$ (1:1 and $CHCl_3$)	cellulose	MATTOX AND MASON [115]
	formamide–water (1:1 or 4:1)	benzene or benzene–isooctane (3:2)	Celite 545	separation incomplete BANES [159]
Corticosteroid acetates	ethanol	petroleum ether–CH_2Cl_2 (1:1) + 0–2% ethanol (gradient)	Florisil	ROSSELET AND LIEBERMAN [132]
	methanol	petroleum ether–CH_2Cl_2 (1:1) + 1% methanol	Florisil	
	methanol–water (4:1)	petroleum ether–benzene (3:2 and 2:11)	Celite 545	separation of aldosterone diacetate from cortisone monoacetate AYRES *et al.* [160]
Corticosteroid acetates, 17-Ketosteroids 20-Ketosteroids	ethanol	1–2% ethanol in CH_2Cl_2, or 1% ethanol in petroleum ether–CH_2Cl_2 (1:1)	SiO_2	KATZENELLENBOGEN *et al.* [161, 162]
	formamide	cyclohexane–benzene (1:1)	SiO_2	
17-Ketosteroids	nitromethane	3% $CHCl_3$ in petroleum ether	SiO_2	JONES AND STITCH [138]

TABLE 21

PARTITION CHROMATOGRAPHY OF ESTROGENS

Stationary phase	*Mobile phase*	*Support*	*Remarks*	*References*
2.3 *N*-NaOH	benzene	Celite 535	separates only estradiol from estrone; estriol can also be eluted if CO_2 is passed through	SWYER AND BRAUNSBERG [163] STERN AND SWYER [164] BITMAN AND SYKES [165]
0.4 *N*-NaOH	benzene	Celite 545	separation of estradiol, α-dihydroequilin and dihydroequilenin	HAENNI *et al.* [166]
3.1 *N*-NaOH	benzene–petroleum ether (4:1) and $CHCl_3$–butanol (17:3)	Celite 535	for the separation of estrone, estradiol, estriol	BRAUNSBERG *et al.* [144]
Benzene	methanol–water (2:8) (4:6) (6:4)	rubber	estriol estradiol (reversed phase!) estrone	NYC *et al.* [141] BOSCH [167]
0.8 *N*-NaOH	benzene ethylene chloride–benzene (3:1)	Celite 535	estrone estradiol	BAULD [168]
Methanol–water (7:3)	ethylene chloride	Celite 535	estriol, separation of 16-*epi*-estriol	BAULD [134] MARRIAN AND BAULD (169]

likewise be separated quite well, although in this case adsorption chromatography is at least equally good, and easier to carry out. Nonetheless, a whole series of relatively weakly polar steroids can be separated by the method of JONES AND STITCH [138] with nitromethane as the stationary phase. Under certain particular conditions, the mid-point of the elution of the following compounds can be given [138] as:

	ml
Cholest-4-en-3-one	24
3β-Chloro-androst-5-en-17-one	33
3,5-Cyclo-6-hydroxyandrostan-17-one	90
Androsterone	90
3β-Hydroxy-5α-androstan-17-one	130
3β-Hydroxyandrost-5-en-17-one	170
3α-Hydroxy-5β-androstan-17-one	210
3α,11β-Dihydroxy-5β-androstan-17-one	210
Androst-4-en-3,17-dione	270

The individual bands extend symmetrically over a range of 30–40 ml.

The usefulness of a system is determined not by its effectiveness in separating synthetic mixtures, but only by applying it to natural extracts, which are usually very impure. It is rarely possible to resolve a random extract of urine or of an organ by means of a single partition chromatogram; the best chances for success lie in successive chromatography in various systems or in combination with adsorption chromatography.

An example of reversed phase chromatography is the defatting of corticosteroid extracts on Hyflo-Supercel-silicone columns; the fat remains in the stationary phase (petroleum ether) while the corticosteroids are eluted with the mobile phase (ethanol–water 1:1) [151].

(c) *Estrogens*

Table 21 (p. 137) presents some of the experiments which have been made on the partition chromatography of estrogens. There is as yet no simple and reliable method for separating the estrogens. It is true that synthetic mixtures can be resolved, but extracts do not yield the pure eluate fractions necessary for quantitative determinations. The gradient-elution technique, and combinations of partition- and adsorption chromatography would undoubtedly be useful.

(d) *Glycosides and aglycones*

This class of substances presents a particularly fine field for the application of partition chromatography. Many mixtures which were formerly difficult to separate, or which are liable to decompose, can be separated well. The first investigations in this field were undertaken by STOLL and co-workers [131], for the separation of cardiac glycosides from *Scilla maritima*, L. Using cellulose (cotton linters) or diatomaceous earth as support, water as the stationary phase, and ethyl acetate or ethyl acetate–butanol (9:1) or ethyl acetate–methanol (95:5) as the mobile phase, they were able to isolate 8 new glycosides. The authors give a very detailed description of these separations.

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The same group of workers also succeeded in separating glycosides and aglycones of the Scilla-, Digitalis-Strophanthus group [129], using a system of silica gel–water–ethyl acetate with 0.5% methanol or 2% methanol in chloroform (*cf.* the original reference for elution curves). REICHSTEIN and co-workers [135], starting in 1953, then employed a similar system with kieselguhr–water as stationary phase and benzene, chloroform, butanol, or mixtures of them as the mobile phase, for the very successful separation of glycosides from strophanthus and other plants; they have described the procedure very precisely. As an example of the separation of a very complicated mixture from the seeds of *Strophanthus vanderigstii*, Staner, one may refer to more recent work by these authors [87], in which numerous partition- and adsorption chromatographic procedures were combined, leading to the isolation in crystalline form of 8 of the 19 Raymond-positive substances known to be present.

For the use of formamide–water (2:1) on Celite 545 and benzene–chloroform (9:1) for the detection of digitoxin in tablets, *cf.* [170].

(*e*) *Bile acids*

Systems with reversed phases have been successfully employed since 1951, especially by BERGSTRÖM AND SJÖVALL [142, 172, 173] for the partition chromatography of bile acids. The stationary phase is chloroform–heptane (9:1) or similar mixtures on hydrophobic Hyflo Supercel (dimethyldichlorosilane), and the mobile phase is 50–60% aqueous methanol. Cholic acid, desoxycholic acid and lithocholic acid can be well separated. The effect of altering the mobile and stationary phases upon the elution volumes of these acids has been carefully investigated, with the aim of finding the optimal conditions. These systems are also suitable for separating keto- and hydroxycholanic acids [172, 173].

NORMAN [174, 175] has succeeded in separating a series of conjugated bile acids. In contrast to the above procedure, he used the usual type of partition chromatography with water as the stationary phase and amyl alcohol–chloroform (51:9) as the mobile phase. The order of elution is then exactly reversed; the procedure with reversed phases seems to be more useful, however. Another "normal" system [171] employs 70% acetic acid on Celite 545, and petroleum ether, petroleum ether–isopropyl ether (6:4) and (4:6). The order of elution is lithocholic acid, desoxycholic acid, α-hyodesoxycholic acid, cholic acid, and dehydrocholic acid.

(*f*) *Related compounds*

Glucuronides of urinary ketosteroids can be chromatographed by the method of EDWARDS AND KELLIE [136] on silica gel charged with 0.1 *N* sodium acetate. On elution with chloroform containing 2% of ethanol and 0.4% of acetic acid, one obtains in succession the glucuronides of androsterone, hydroxy-5β-androstanone, testosterone and, on raising the acid content to 5%, the 11-hydroxyketosteroids.

Steroid alkaloids (veratridine, cevadine) can be separated well if silica gel and

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citrate buffer pH 4.25 are used as the stationary phase, and chloroform, as the mobile one [176].

9. Concluding remarks

The use of partition chromatography in the field of steroids could be developed much further, because of the relatively large range of possibilities for varying the solvent systems and because various systems can be employed one after the other, thus markedly improving the separations. Practically no side reactions between steroids and supporting media have yet been observed; only MATTOX AND MASON [115] have reported an unfavorable effect of silica gel and formamide, but other authors have not found this. Small losses are of course inevitable in the course of partition chromatography, but they should not exceed 10%.

As far as the relationship between constitution and rate of migration or order of elution is concerned, much more can be said in the case of partition chromatography than in that of adsorption chromatography; the relative position of a substance in the column depends essentially upon its distribution coefficient in the given system, provided that other effects such as adsorption, chelation, etc. do not play a large part. In normal partition chromatography therefore, a substance moves faster, the less polar it is; the more polar it is, the more slowly it moves. In reversed phase chromatography, exactly the opposite is the case.

For the discussion of the finer differences in migration rates we refer to the corresponding section on paper chromatography, since better possibilities for comparisons appear there. There are no differences in principle, however.

4. PAPER CHROMATOGRAPHY

Paper chromatography, which was introduced in 1944 by CONSDEN, GORDON AND MARTIN [183] was in its original form beautifully adapted to the separation of amino acids, sugars and other water-soluble materials, but not of strongly lipophilic substances like sterols and steroids. It was indeed possible to perform group separations by chromatography of water-soluble derivatives like, for example, Girard-hydrazones [190], but such methods proved unsuitable for general use, because of the necessity for carrying out a chemical reaction, and because of inadequate separations. The sterols and steroids became accessible to paper chromatography only with the introduction of special solvent systems by ZAFFARONI *et al.* [191] in 1950, and by BUSH [177] in 1952, as well as numerous variations of them. Thanks to its simplicity and effectiveness, this technique has in the last few years developed to an unforeseen degree and is now applicable in practice to all types of steroids. The ingenious application of these methods has at the same time markedly aided progress in all the steroid fields, be they microbiological reactions, isolation and determination of hormones, phytochemistry, biosynthesis, or purely chemical synthesis. Paper chromatography has served not only as a qualitative and quantitative analytical procedure for microquantities of material, but has also been employed on a preparative scale. For the

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theory and practice of paper chromatography in general, we refer to the literature [2, 80, 106, 118, 182]. Special applications to steroids have already been dealt with in several reviews [103, 105, 150, 179, 180, 181].

The pertinent literature as well as the author's own experience will be discussed below; specific examples from all important classes of steroids will be adduced, and the connection between constitution and chromatographic behavior will be investigated.

1. Procedures

The chromatography of steroids on paper is essentially partition chromatography, and those concepts are valid which have already been discussed in Section 3 B 1, p. 129. Adsorption phenomena often enter in, however, playing a greater or smaller role, depending upon the nature of the solvent system and the material being chromatographed. Undesirable adsorption effects (tailing) can be largely avoided by chosing a system in both of whose phases the substrate is more soluble; among other ways, this can be achieved by working at an elevated temperature.

(*a*) *Solvent systems*

A large choice of suitable systems is available for nearly all classes of steroids. These systems can be classified in the following way (all compositions are volume/volume ratios):

(1) ZAFFARONI-type systems (usually free of water). The paper is first impregnated with the stationary phase, then loaded with the substance, and the mobile phase is added last. Table 22 surveys such systems, which can be chosen to suit all classes of steroids. The two phases can be used in any combination, provided that they are not infinitely miscible with one another. Some of the most commonly used systems are

TABLE 22

ZAFFARONI-TYPE SOLVENT SYSTEMS

Stationary phase (for impregnating the paper)	*Mobile phase (saturated with stationary phase)*
Propylene glycol	hexane, cyclohexane
Propylene glycol–water	methylcyclohexane
Propylene glycol–ethylene glycol	petroleum ether, ligroin
Ethylene glycol	decalin
Cellosolve (ethylene glycol-monoethyl ether)	benzene, benzene–cyclohexane, benzene–chloroform
Phenylcellosolve (ethylene glycol-monophenyl ether)	benzene–ethyl acetate, dichlorobenzene
Carbitol (diethylene glycol-monoethyl ether)	xylene, xylene–methyl ethyl ketone
	methylene chloride
1,3-Butanediol	chloroform
Glycerol	chloroform–tetrahydrofuran
Formamide	acetone
Benzyl alcohol	methyl ethyl ketone
Water	butanol, amyl alcohol

References p. 186.

for example propylene glucol/toluene, formamide/benzene or benzene–chloroform and chloroform.

(2) BUSH-type systems (aqueous). The dry paper, charged with the substance, is hung in a chromatography chamber which is saturated with both phases, and allowed to equilibrate for a time. The paper preferentially adsorbs the polar phase, and the chromatogram is then developed with the weakly polar mobile phase. Table 23 presents a survey of such systems, which are also suitable for most neutral steroids, preferably at elevated temperatures (38–40° C).

TABLE 23

BUSH-TYPE SOLVENT SYSTEMS

Stationary phase	*Mobile phase*
Methanol–Water *t*-Butanol–Water	hexane, cyclohexane, heptane, isooctane, petroleum ether, benzene toluene, isopropyl ether, ethyl acetate

Examples of systems of this type which are frequently employed are:

Bush A (heptane–methanol–water 5:4:1)
B_5 (benzene–methanol–water 2:1:1)
C (toluene–ethyl acetate–methanol–water 9:1:5:5)
E_2B (isooctane–*t*-butanol–water 10:5:9)

(3) Aqueous systems in the classical sense, without special techniques for impregnating or equilibrating, used especially for strongly polar or ionic steroids. Tables 24–26 present a selection of neutral, acid and basic systems.

Monophasic systems are also used on occasion, as for example the water-free system benzene–chloroform–acetic acid (100:4–40:1–4) for sapogenins [370]. It is of course true that biphasic systems are produced on the cellulose.

(4) Systems for reversed phase chromatography. These are useful for various types of steroids, often for the very weakly-polar ones. Table 27 presents a survey of the possibilities of such systems. The cellulose must first be rendered hydrophobic by impregnation with various materials, so that the less-polar phase can be the stationary one.

TABLE 24

AQUEOUS NEUTRAL SOLVENT SYSTEMS

for use, *e.g.*, with cardiac glycosides and aglycones, saponins, sapogenins, aminosteroids, etc.

Stationary phase	*Mobile phase*
Water	isooctane, ligroin
Methanol	benzene, toluene, xylene
Ethanol	chloroform
Butanol	carbon tetrachloride
Isoamyl alcohol	butanol and higher alcohols
	dioxane

References p. 186.

TABLE 25

AQUEOUS ACID SOLVENT SYSTEMS

for use, *e.g.*, with bile acids, steroid carboxylic acids, steroid conjugates, saponins, steroid alkaloids, etc.

Stationary phase	*Mobile phase*
Formic acid–water	heptane
Acetic acid–water	toluene
Hydrochloric acid–water	isopropyl ether, butyl ether
(with or without addition of	carbon tetrachloride
alcohols, on plain or KCl-	ethyl acetate
impregnated paper)	alcohols, esp. butanol

TABLE 26

AQUEOUS ALKALINE SOLVENT SYSTEMS

for use, *e.g.*, with estrogens, bile acids, steroid carboxylic acids, steroid conjugates, saponins, etc.

Stationary phase	*Mobile phase*
Water	benzene, toluene, xylene
N-NaOH	chloroform
Dil. ammonium hydroxide	isoamyl alcohol
Ethanolamine	pyridine, collidine (+ NH_3)
Carbonate buffer	
Sodium barbital buffer	

TABLE 27

SYSTEMS FOR REVERSED-PHASE CHROMATOGRAPHY

Cellulose rendered hydrophobic with	*Stationary phase*	*Mobile phase*
Vaseline	alcohol	water [192]
Paraffin oil,	methanol	water [193]
Kerosine	ethanol	water [194]
(5–10% in petroleum ether)		dil. ammonia [194]
	n-propanol	water [195]
		methanol, water [193]
	cellosolve	propanol, methanol [193]
	chloroform	acetic acid [196, 197]
Petroleum	alcohols	water [198]
(10–40% in petroleum ether)		
Silicone	hexane, benzene	methanol, water [199, 200]
(5–10% in cyclohexane)	cyclohexane	ethanol, water [201]
	chloroform	methanol, water [203]
Aluminum soap	carbon tetrachloride	methanol, water [204]
Quilon	methyl cellosolve	alcohols, water [202, 205]
	pentanol, octanol, esters of dicarboxylic acids	formamide, water [248]
	heptanol	water [206, 207]

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It is obviously essential with all these systems that all immiscible phases be mutually saturated at the temperature of the experiment.

For purposes of orientation in this complicated field, Fig. 4 presents the R_F-values of 20 steroids (tetrahydrocortisol to cholesterol) whose polarity decreases from top to bottom, in 14 proven "standard" solvent systems whose polarity increases from left to right (phenyl-cellosolve/heptane to E_2B). Among other things, this scheme permits one to make a rapid choice of a suitable system for a steroid whose polarity is approximately known (from the number of hydroxy- and keto groups and double bonds). Another scheme is presented in Table 28, for ZAFFARONI-systems [178]. Table 29, on the other hand, lists a selection of BUSH-systems [177, 181]. Their applicability can be determined by comparison with the BUSH-systems presented in Fig. 4.

The relative R_F-values of the steroids are for the most part the same in the various systems, since they depend primarily on the polarity of the steroids. There are however always cases in which some of the individual R_F-values in certain systems are shifted by the effects of adsorption, displacement, or hydrogen bonding; this phenomenon is particularly valuable for the characterization of steroids. It is therefore unreservedly advisable to employ ZAFFARONI-systems as well as BUSH-systems.

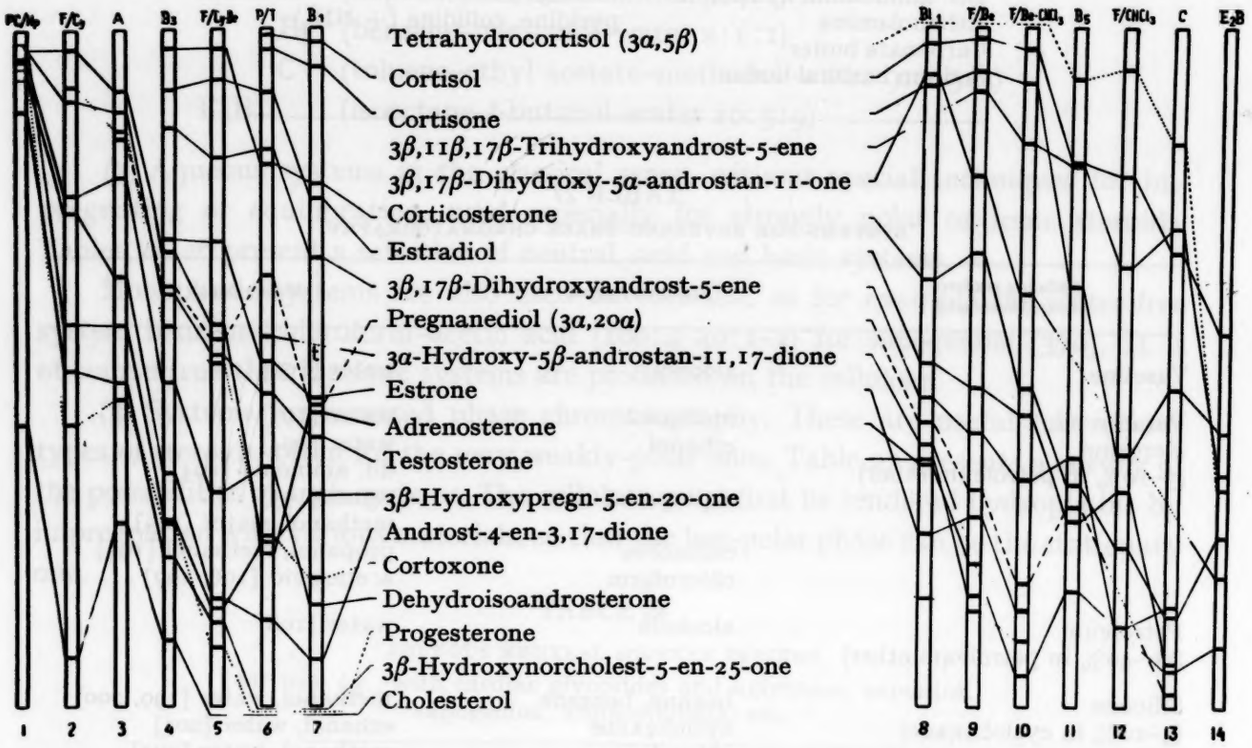

Fig. 4. Average R_F-values of 20 steroids whose polarity decreases from top to bottom, in 14 "standard" solvent systems, whose polarity increases from left to right. (Temp. 22° C, Whatman Paper No. 1, ascending chromatogram). t = tailing. 1. phenylcellosolve/heptane; 2. formamide/cyclohexane; 3. Bush A (heptane–methanol–water 5:4:1); 4. Bush B_3 (petroleum ether–benzene–methanol–water 33:17:40:10); 5. formamide/cyclohexane–benzene (1:1); 6. propylene glycol/toluene; 7. Bush B_1 (petroleum ether–toluene–methanol–water 5:5:7:3); 8. petroleum ether–benzene–methanol–water (3:7:5:5); 9. formamide/benzene; 10. formamide/benzene–chloroform (1:1); 11. Bush B_5 (benzene–methanol–water 2:1:1); 12. formamide/chloroform; 13. Bush C (toluene–ethyl acetate–methanol–water 9:1:5:5); 14. Iso-octane–*t*-butanol–water (10:5:9).

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TABLE 28

ZAFFARONI-SYSTEMS FOR VARIOUS POLAR STEROIDS [178]

Solvent	$C_{19}O_2$ $C_{21}O_2$	$C_{21}O_3$	$C_{21}O_4$	$C_{21}O_5$	$C_{21}O_6$	$C_{21}O_3$	$C_{21}O_4$	$C_{21}O_5$	$C_{21}O_4$	$C_{21}O_5$
						21-Monoacetates			*Diacetates*	
Formamide/hexane	×	×				×			×	
Propyleneglycol/hexane	×	×				×			×	
Formamide/hexane–benzene (1:1)	×	×	×				×	×		×
Propylene glycol/hexane–benzene (1:1)	×	×	×				×	×		×
Formamide/benzene		×	×	×				×		
Propylene glycol/benzene		×	×	×				×		
Propylene glycol/toluene		×	×	×				×		
Formamide/chloroform			×	×	×					

TABLE 29

BUSH-SYSTEMS OF VARIOUS POLARITIES [177, 181]

Petroleum ether–methanol–water	(100:96:4)	
Petroleum ether–methanol–water	(100:85:15)	
Petroleum ether–methanol–water	(100:80:20)	= A
Petroleum ether–methanol–water	(100:70:30)	
Petroleum ether–toluene–methanol–water	(25:25:35:15)	= B_1
Petroleum ether–toluene–methanol–water	(33:17:30:20)	= B_2
Petroleum ether–benzene–methanol–water	(33:17:40:10)	= B_3
Toluene–methanol–water	(100:75:25)	
Toluene–methanol–water	(100:50:50)	= B_4
Benzene–methanol–water	(100:55:45)	
Benzene–methanol–water	(100:50:50)	= B_5
Benzene–methanol–water	(100:40:60)	
Toluene–ethyl acetate–methanol–water	(90:10:50:50)	= C
Benzene–chloroform–methanol–water	(66:33:50:50)	
Benzene–chloroform–methanol–water	(50:50:50:50)	
Xylene–methanol–water	(100:85:15)	
Xylene–methanol–water	(100:70:30)	

	Solvent systems, type	
	Zaffaroni	*Bush*
Loading capacity	greater	smaller
Suitability for preparative chromatoblocks	very good	not possible
Displacement effects	sometimes present	not observed
Sensitivity to temperature fluctuations	moderate	considerable, higher temperatures are usually necessary
Drying of the chromatograms	time-consuming at room temperature, otherwise higher temperatures are advisable; the last traces of the stationary phase are difficult to remove from the paper	very easy at room temperature

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Several differences between the two types which are of practical importance will be pointed out below. As has been emphasized in earlier sections, all the solvents must be quite pure, and of constant quality. For purification (washing to neutrality, distillation, etc.,) *cf.* 3 A 3, p. 108. Various solvents "for chromatography" are available commercially (*e.g.*, formamide, Merck) and can be used without further purification. All solvent systems must be freshly prepared at not too great intervals. Chemical changes are possible in some cases.

(*b*) *Paper*

Quite a number of suitable filter papers are available. Besides the Whatman No. 1 and Schleicher and Schüll 2043b (*cf.* [209]) which are probably the most-frequently employed, many other types of paper have proved useful for steroid chromatography (in part, for special purposes). These include, for example, Whatman No. 2, 3 MM, 4, 7, 31, 54, 542, Schleicher and Schüll 2040 b, 2043 a, Munktell 20, 150 G, Eaton and and Dikeman 613, Durieux 122, d'Arches 1030, Marcherey and Nagel 63. Separated strips can be used, or strips joined together, as in the original work of ZAFFARONI *et al.* [191] (*cf.* also [210, 211]) and as shown in Fig. 5 and 6. Entire sheets may also be used, in which case the dimensions are determined on the one hand by the size of the original sheets, and on the other, by the size of the chromatography cabinets. Strips have the advantage that lateral diffusion is suppressed, so that the distance between substances which are to be chromatographed in parallel can be smaller than on sheets. On the other hand, disturbing edge effects can arise when the strips are heavily loaded. For analytical purposes we always employ, with considerable success,

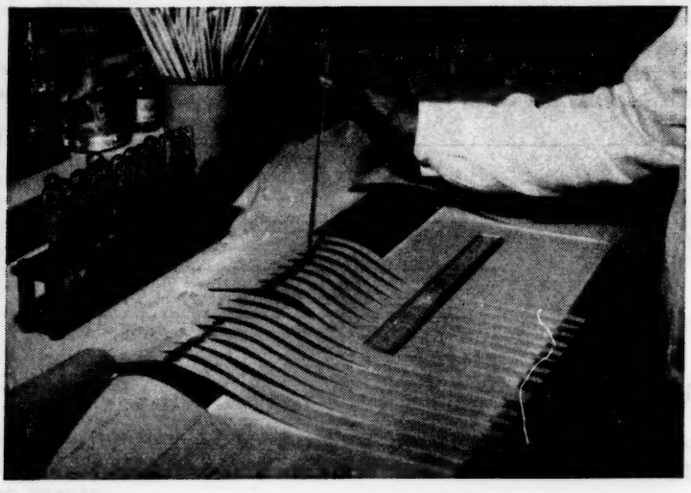

Fig. 5. Application of the dissolved materials to strips and to sheets.

stamped-out strips of Whatman No. 1, 1.5 cm wide (for the other dimensions, *cf.* [210] and Fig. 5). For preparative chromatograms we use sheets 19 cm wide, loading the paper along a line 17 cm wide, and developing the chromatogram perpendicular to the length of the roll as it was formed in the paper-making machine.

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The paper is not usually washed before being used for chromatography. For some preparative purposes, or especially when the eluate from the chromatogram is to be

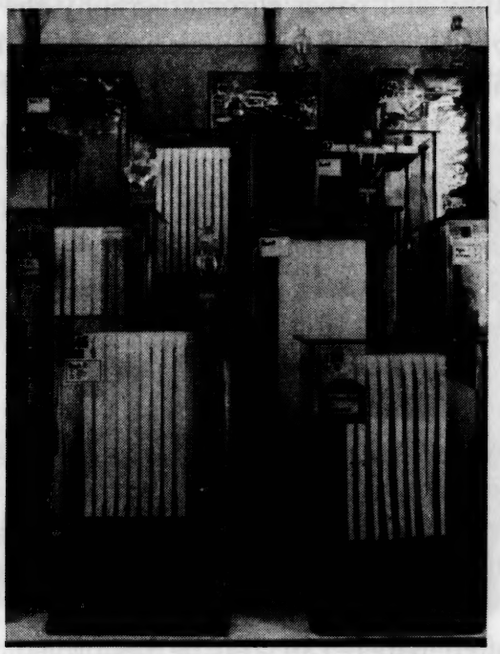

Fig. 6. Complete vessels for paper chromatography.

evaluated quantitatively, the papers are washed either with the solvent system which is to be used or with individual solvents; the wash liquid is run through the paper for several days, or a Soxhlet extractor is used (*cf.* [210, 212]).

Pretreatment of the papers

(1) For systems of the ZAFFARONI-type (Table 22) it is necessary to impregnate the paper with the stationary phase. This is most easily accomplished by drawing the paper rapidly through a 20–50% solution of the impregnating material in methanol, acetone, or chloroform. After hanging for 5–10 minutes (apparently it makes no difference in what direction; it is best to do it in a hood) the volatile solvent has evaporated (at high humidities care must be taken to prevent too much condensation of water as a result of cooling when the solvent evaporates) and the paper is ready for application of the substances. This operation should not take too long (less than half an hour); otherwise too much of the impregnating material will evaporate. Such a decrease in the volume of the stationary phase increases the R_F-values. On the other hand, if the paper is dipped into undiluted impregnating medium and then pressed out between hard rubber rollers or filter paper, there is usually so much stationary phase present that the materials move only very slowly. The structure of the paper must be taken into account, too, in this connection; dense papers take up less of the stationary phase than do loosely-woven ones [209]. For chromatograms of the

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ZAFFARONI-type we always use 20 or 30% solutions of the impregnating medium in acetone [213].

(2) Impregnation with adsorbents. In an attempt to combine the advantages of adsorption chromatography and paper chromatography the paper was impregnated either with alumina [177, 214, 215] or with silicic acid [192], but all the advantages of partition chromatography were lost thereby. The separations are generally not convincing, and difficultly reproducible, so that this subject will not be pursued further at this point.

(3) Rendering the paper hydrophobic. The object of this measure is to obtain reversed-phase chromatography, with the non-polar phase stationary, and the polar one, mobile (*cf.* 3 B 1, p. 129), for use, *e.g.*, with extremely lipophilic steroids. It is most simply accomplished by impregnation with paraffin oil, petroleum, vaseline or silicones (*cf.* Table 27). Silicone-impregnated papers are obtainable commercially (Schleicher and Schüll 2043 a Mgl hy and 2043 b Mgl hy). Higher alcohols, aluminum soaps, quilon (chromic acid–stearic acid complex) or rubber are also used occasionally; these special systems seem to have a very limited field of application, to be sure.

A further possible way of rendering the paper hydrophobic is to acetylate it [216]. This is relatively complicated, and the commercial acetylated papers still appear to be unsatisfactory. Most of the separations in the steroid field which can be carried out with the aid of acetylated paper, can also be performed more conveniently with ordinary paper and suitable systems (*cf.* however [389]).

(*c*) *Application of the substance*

The quantity of substance used depends upon the purpose of the chromatography, the solubility of the substance in the solvent system used and the ease of detection of the material on the chromatogram. For the usual paper chromatographic analysis it is best to use 5–20 μg of material, dissolved in 5–10 μl of solvent which is dripped onto the starting point (diameter 5–15 mm) with a micropipette. If the substance is easily detectable, one can operate with quantities down to 0.25 μg; if it is easily soluble, with quantities up to about 1000 μg per 1.5 cm of strip-width. Small quantities of material naturally permit better separations, but one is often forced to use larger quantities in order to detect minor impurities. For preparative chromatography that quantity of material is applied per cm along the starting line which an analytical pilot experiment has shown to be best with respect to separation and loading capacity. To drip the solution onto the paper, the latter is laid over a U-shaped wooden framework in such a way that the point of application lies over the empty space (Fig. 5).

Solvents for the materials to be applied may be, among others, acetone, chloroform, methanol, or, very advantageously, chloroform–methanol (1:1). Standard solutions are best stored in a not-too-volatile solvent like methanol, at a concentration of 0.2–1%, in tubes with ground glass stoppers (see Fig. 5). They should be kept in the cold, and stored not longer than a few weeks.

The micropipettes should have a capacity of 50–100 μl, and be graduated in μl.

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For special purposes one can use exactly calibrated ultramicropipettes or, for example, an Agla micrometer syringe, although this consumes a considerable amount of time. The application for an analytical chromatogram is most simply made in the form of a point, and that for preparative chromatograms in the form of a line; one allows the contents of a 100 μl pipette to flow out uniformly along a 17-cm starting line, for example. Blowing air against the bottom side of the starting place in order to hasten

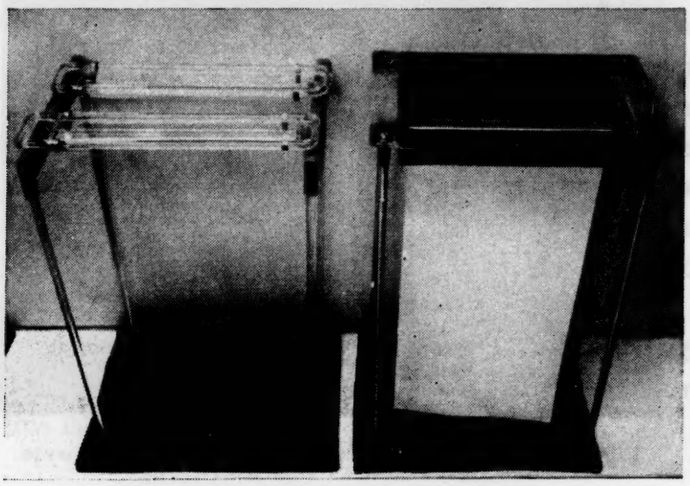

Fig. 7. Troughs of glass and stainless metal.

the drying is usually not necessary with organic solvents. For a special method of applying large volumes (which is, however, of only limited applicability) *cf.* [177]; for such a method combined with an initial purification, *cf.* [217].

It is usually advisable to run known standard substances such as steroids, dyes or other compounds along with the mixtures, fractions or substances to be analysed, and also to include a strongly polar dye like Sudan III or IV, which runs with the solvent front and marks it clearly.

(*d*) *Conditions for chromatography; apparatus*

Ascending or descending chromatograms may be run; the descending technique is more advantageous, since it permits the running of the chromatogram over whatever distance is desirable or necessary by varying the duration of the experiment from a few hours to several days. All chromatograms should be developed at as constant a temperature as possible, especially when BUSH-systems are used. All solvent systems are usable with the containers usually used for paper chromatography; they must be resistant to the action of the solvent, and capable of being closed tightly. They should not have too great a volume relative to the surface area of the chromatogram, since this renders it difficult to saturate with the vapors of the solvents.

Satisfactory results are obtained with, for example, the glass containers (rectangular battery jars) shown in Fig. 6, which are simple and easy to see into. They have a ground edge and matching ground covers. Silicone vacuum grease is used for the

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seal. Another very simple arrangement (Fig. 7) has glass or metal troughs (in exactly horizontal position) in which the prepared paper strips or sheets are hung. The metal troughs of stainless steel (with 10 slots [218]) shown in Fig. 7 have the advantage of taking up very little space (100 strips or 10 sheets per trough). When BUSH-type

Fig. 8. Warm-room with air circulation and drawers.

systems are used, the papers, with the substances applied, are hung in the dry trough, and equilibrated 6–12 hours in the closed container, which is saturated with solvent vapors (the time of equilibration depends, among other things, upon the amount of paper). The saturation of the vessels is hastened by papers at the sides, one of which is saturated with the stationary phase, and the other, with the mobile phase. When the equilibration is complete, the necessary quantity of the mobile phase is allowed to flow from a dropping funnel into the trough, through an opening in the cover of the container, and the chromatography then begins. After 2–3 hours the solvent front has reached the end of the paper. At this time, or occasionally even later (in which case the eluate can be collected) the chromatogram is removed and hung up to dry.

The equilibration and chromatography must be carried out at the same temperature; with BUSH-systems smaller spots with more definite boundaries, and hence better separations, are usually obtained at 35–40° C than at room temperature. Chromatography at elevated temperature is carried out in incubators, special heated cabinets, or warm-rooms. For more hygienic and more certain operation, a special chest of drawers with warm air circulation has proved excellent, even for large numbers of chromatograms (Fig. 8, [36]).

With ZAFFARONI-type systems the paper is charged with stationary phase and the substances to be separated, and placed directly in the trough, which has been filled with the mobile phase; the chromatography begins immediately without equilibration in the cabinet, which has been saturated with mobile phase. For an arrangement for ascending chromatography on paper *cf.* for example [219]. This

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somewhat cumbrous procedure would probably be more or less out of the question for general use. For a horizontal arrangement, *cf.* [392].

After the desired interval of time, the finished chromatograms are removed from the flask and hung up by the bottom or the top edge. They are protected from light, especially UV [220, 221] and allowed to dry at room temperature, preferably in a hood. Unimpregnated papers are sufficiently dry in $^1/_2$–1 hour; impregnated papers are either allowed to dry for 12–14 hours in a gentle stream of air, or, after a short preliminary drying in the air, they are held in a drying oven for 20–40 minutes at 80–90° C, while the vapors are sucked off. Remains of the impregnating materials always cling stubbornly to the paper. Although chromatograms which are to be examined by color reactions must be dried as thoroughly as possible, since residues, especially formamide, can often impede color-development or fluorescence, this is not usually necessary for chromatograms from which the materials are to be eluted. On the contrary, the elution is easier if the paper is not completely dry; the impregnating material which remains can be relatively well removed *in vitro* by a high vacuum. For the exceptional substances which are sensitive to oxygen, the chromatograms can be dried in a heated chamber through which a stream of inert gas is passed [179], but this is usually tedious.

(*e*) *Test reactions; evaluation of the chromatograms*

The discovery of a test reaction is just as important as a good solvent system for the paper chromatography of the colorless steroids. Fortunately, a large number of excellent indicators or reagents for this purpose are already known, so that almost all classes of steroids can be detected.

There are indeed various indicators which are specific for individual chemical groups, but there are of course none which are strongly specific for one or more of the steroids. A compilation of useful test reactions for steroids and related substances is given below. It should always be borne in mind that certain steroids or impurities are

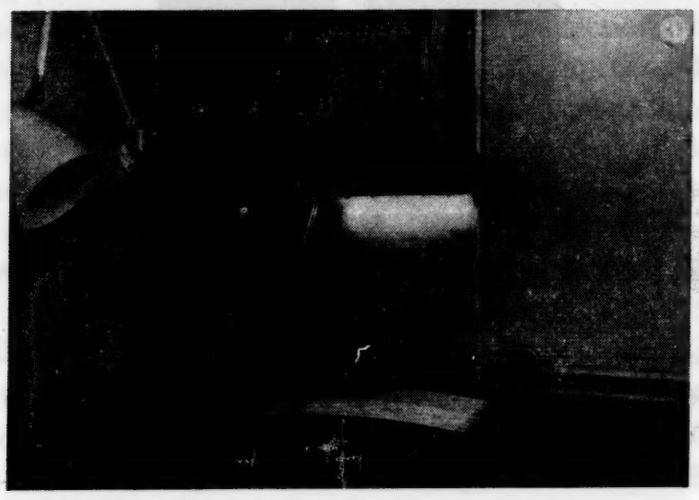

Fig. 9. Apparatus for making UV-photocopies.

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sometimes identifiable without any treatment, simply by their fluorescence in UV-light (*ca.* 360 mμ). When treating with reagents we always prefer dipping the chromatogram (rapid, careful drawing of the paper through a flat dish of reagent) to spraying, if it is at all possible.

(1) *UV-absorption.*

(a) At 240 mμ for steroids with α,β-unsaturated oxo-groups. It is most advisable to make a copy in UV-light before carrying out any color reactions if UV-absorbing steroids are suspected [46, 139, 149, 177, 222, 223, 224] or to examine them with a fluorescent screen [46, 149, 218, 224]. The light source for the photocopy (*cf. e.g.*, Fig. 9) is a germicidal lamp with its principal emission at 253.7 mμ. The chromatogram is stretched over a suitable, very hard photographic paper (Kodagraph Contact Standard Paper, Ilford reflex paper No. 50, Agfa Copex C, Tellko, etc.) with the aid of a polyethylene sheet, if necessary. An exposure of a few seconds is followed by development and fixing, and the permanent record reveals the UV-absorbing steroids as white spots or bands on a dark background (Fig. 10). A somewhat more elaborate arrangement for exposing is given in [222]. The sensitivity is about 0.5 μg α,β-unsaturated ketosteroid

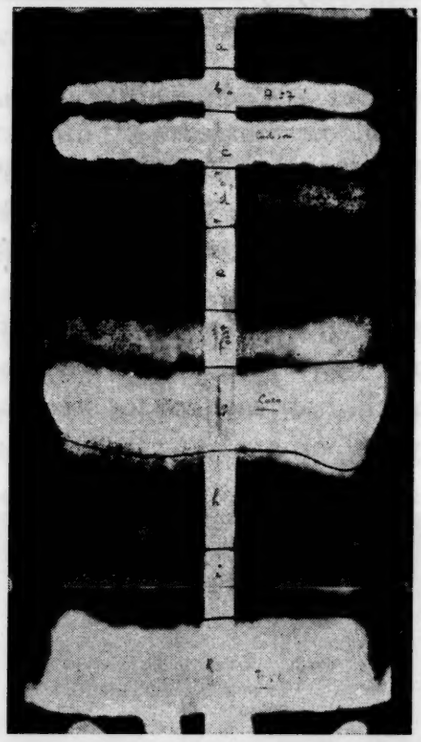

Fig. 10. UV-photocopy of a preparative sheet-chromatogram.

per cm^2. A fluorescent screen (see Fig. 11, [218]) serves the same purpose. It has, instead of the photographic paper, a screen which fluoresces green on illumination at 253.7 mμ. The UV-absorbing steroids on the chromatogram placed between the light source and the screen cause dark places on the screen, which can be marked directly on the chromatogram from the rear.

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(b) Filtered light from a mercury vapor lamp is used for making UV-photocopies at 300–340 mμ for the lactone ring of scilla- and bufo-glycosides [225] and aglycones; for estrogens at 280 mμ, magnesium light may be used, for example [226].

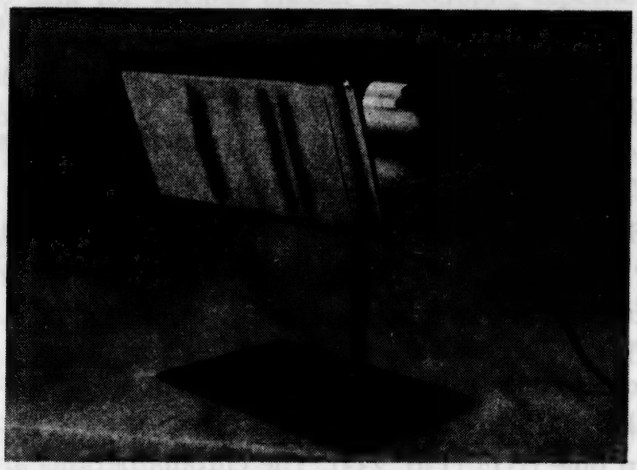

Fig. 11. Fluorescent screen with chromatogram.

(2) *m-Dinitrobenzene (DNB) and alkali* (Reaction of ZIMMERMANN or RAYMOND) for 17-ketosteroids, cardiac glycosides, and aglycones (butenolides).

(a) The strips are drawn through a freshly-prepared mixture of equal parts of (1) 3 g DNB in 190 ml methanol + 10 ml propylene glycol and (2) 2.5 g KOH in 20 ml methanol + 5 ml water [227].

(b) 2.5% DNB in chloroform: dip, dry, then spray with 5% KOH in methanol [228].

(c) 10% DNB in benzene: spray, then spray with a solution of 6 g NaOH in 25 ml water and 45 ml methanol [229].

(d) Freshly-mixed solution of 1 vol. 2% DNB in ethanol + 1 vol. 30% KOH in ethanol [230] or 2 vol. 1% DNB in methanol + 1 vol. 15% KOH [231].

(e) 2.5 *N* KOH in ethanol: dip, then dip into a solution of 2% DNB in ethanol, dry at 65° C [232].

17-Keto compounds give a violet color (limit of detection, about 2–5 $\mu g/cm^2$). Other colors, much weaker, with 3-ketones (blue–blue violet), 16-ketone (yellowish-brown [230, 232]), 20-ketones (brownish-violet [232]), more intense with Δ^4-3,6-diketones (orange yellow–blue green–brown green; specific?) [180]. When certain solvent systems have been used, the chromatograms show a tendency to turn brown [89].

(3) *2,4-Dinitrophenylhydrazine (DNPH)* for steroids with reactive keto-groups [324].

(a) Strips drawn through a solution of 3 g DNPH in 900 ml methanol + 3 ml conc. HCl; dried 5 min at 90°, washed with 2 *N* NaOH, 2 *N* HCl and water (3 times) [36].

(b) 300 mg DNPH in 5–6 ml ethanol + 0.3 ml conc. H_2SO_4, heat until dissolved, dilute to 200 ml with ethanol, spray [150].

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(c) 150 mg DNPH in 25 ml water + 22 ml conc. HCl, dilute with water to 100 ml [231].

(d) 1 g DNPH in 1 l ethanol + 10 ml HCl [230].

The DNPH reagent can be combined with Tollens reagent on the same chromatogram [150]. The order of sensitivity is Δ^4-3-ketones (2–5 $\mu g/cm^2$; orange) > 3-ketones (yellow) > 20-ketones > 17-ketones > 3-keto-1,4-dienes; α-ketols are very poorly indicated, 11-ketones not at all [150, 232].

(4) *Diammine silver* (Tollens reagent) for reducing steroids [190, 191, 233], glycosides and aglycones [234], also α-bromoketones [150].

Dip into a freshly-prepared solution of 10 ml 0.1 *N* $AgNO_3$ + 10 drops conc. NH_4OH + 5 ml 10% NaOH; when the color development is complete, rinse in a 5% solution of $Na_2S_2O_3$ and in water [191].

(5) *Tetrazolium salts* for reducing steroids (α-ketols).

(a) Triphenyltetrazolium chloride [233]: 2 vol. 0.2% in water + 1 vol. 10% NaOH are mixed shortly before use; spray or, better, dip (red spots).

(b) Tetrazolium blue (BT) [139, 233]. The chromatograms are drawn through a fresh mixture of 1 vol. 0.1% BT + 9 vol. 2 *N* NaOH (keeps 1–2 days) and laid on a glass plate. Steroids with α-ketol chains can be detected down to 0.2 $\mu g/cm^2$ (blue spots immediately). Ring α-ketols are also indicated, and sometimes even Δ^4-3-ketones or glycol side chains (though the test is weak and slow to develop). For other reagent mixtures, *cf.* [235]. The BT-test can be combined to great advantage with the NaOH-fluorescence reaction, *q.v.*

(6) *Arseno-molybdate* [236] for steroids with α-ketol or Δ^4-3-keto groups (blue spots). 25 g of ammonium molybdate in 450 ml water + 21 ml conc. H_2SO_4 + solution of 3 g $Na_2HAsO_4 \cdot 7H_2O$ in 25 ml H_2O. Warm 48 hours at 37° and filter. Application is complicated, *cf.* [236].

(7) *Sodium hydroxide-fluorescence* for Δ^4-3-ketosteroids [177]. Best with aqueous NaOH in combination with tetrazolium blue [139, 237] as described under tetrazolium blue. After preliminary drying of the sprayed chromatograms at room temperature on a glass plate for 30–45 min, the papers are dried in an oven at 90° (15–20 min, in a gentle current of air, or with an infra-red lamp). The conditions for obtaining optimal fluorescence must be determined empirically. The reaction is fairly specific for Δ^4-3-ketosteroids (deep yellow fluorescence on irradiation with UV at about 360 mμ) and very sensitive (limit of sensitivity about 0.1 $\mu g/cm^2$). Progesterone and several related steroids, as well as 19-nor-steroids and 2α-hydroxysteroids [238] react more weakly, and multiply-conjugated Δ^4-3-ketones do not react at all. Even 7-hydroxy-Δ^4-3-ketones do not react, because of the ease of formation of 3-keto-4,6-dienes. With 19-hydroxy-Δ^4-3-keto compounds there is a more or less marked displacement of the fluorescence toward the green or blue-green [68]. The Δ^1-3-ketones, and α,β-unsaturated ketones in other positions than C_3 do not appear to react [181]. Saturated 3,6-diketones give a yellow fluorescence, presumably by enolization to Δ^4-3-keto-6-hydroxy compounds.

(8) *Isonicotinic acid hydrazide* (*INH*) has recently been proposed as a reagent for

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Δ^4-3-ketones [239]. After spraying with a solution of 25 mg INH in 50 ml of acidified alcohol (0.625 ml conc. HCl per 1000 ml distilled alcohol) one dries for 1 hour at room temperature.

(9) *Phosphoric acid* for various classes of steroids, especially those containing hydroxyl groups (including corticosteroids [213, 218]), cardiac glycosides, aglycones [215, 240] and bile acids [240]. A vivid fluorescence of varying intensity is obtained with UV-light (about 360 mμ; cortisol fluoresces green down to 1 μg/cm^2). For dipping, one usually uses 20% aqueous solution [213, 218]; for spraying, an alcoholic one [241]. Residues of propylene glycol on the chromatogram enhance the intensity of the color, while formamide diminishes it. Qualitatively similar fluorescence colors are also obtained with 20% *p*-toluenesulfonic acid in ethanol [218].

(10) *Trichloroacetic acid* for pregnanetriols [242], cardiac glycosides and aglycones (*e.g.*, [228, 243, 244, 245, 246]), sapogenins [247] and other substances. Usually used as a spray:

(a) 25% in ethanol [228, 242] or 20–25% in chloroform [245, 248]. Heat 5 minutes to 100°.

(b) 33% in chloroform + 1 drop H_2O_2 per 10 ml of reagent [244].

(c) 2 vol. 25% solution in ethanol + 8 vol. 3% chloramine in water, heat 10 min at 120° [228]. Various fluorescences are usually obtained with UV-light (360 mμ).

(11) *Phosphomolybdic acid* for various steroids (blue spots [195, 249]), steroid sulfates and glucuronides [250], estrogens [200], bile acids [251, 252], steroid alkaloids. 2–10% in alcohol at 80–100° C [249, 250], or 10% in acetone [253]. Different sensitivities (1–50 μg/cm^2).

(12) *Silicotungstic acid* for various steroids [249], but usually of low sensitivity. 25% in ethanol, spray, dry 2–5 minutes at 110°; various colors.

(13) *Phosphotungstic acid* for sterols [195, 197]; 10–15% in ethanol.

(14) *Phenol–molybdate–perchloric acid* for Δ^5-3-hydroxysteroids [254]: Spray with phenol which is saturated with water, dry at 20–75° C; spray with 1 g ammonium molybdate + 2.5 ml 60% perchloric acid in 100 ml 0.1 *N* HCl and heat to 75–80° for 3–8 minutes. Rose color with Δ^5-3β-hydroxy-steroids; sensitive to about 2 μg/cm^2 (not chromatographed).

(15) *Antimony trichloride* for steroids of all classes, especially Δ^5-3-hydroxy-steroids [213], sterols [193], estrogens [255], progesterone-like steroids [256], cardenolides [228, 248, 257, 258], bufogenins [96], sapogenins [259, 260], bile acids [261] and various others [214, 262, 263].

(a) Saturated solution in chloroform [213], dip and dry 1–5 min at 80–90°.

(b) 25 g antimony trichloride in 5 ml nitrobenzene [262], spray and heat to 90°.

(c) 20% in methylene chloride + 2% acetyl chloride [263].

(d) Pretreatment of the chromatograms with chlorine gas for 20 min, then spray with a mixture of 38 g antimony trichloride and 10 ml acetic anhydride (freshly prepared), heat to 90–100° C; in many cases more intense colors or fluorescence are obtained with chlorine treatment than without [214].

(e) Saturated solution of antimony trichloride in chloroform + 10–20% thionyl

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chloride. Spray, heat 3–5 min at 95° [198]; this variation is also said to produce more intense color reactions.

Antimony trichloride reagents are especially advantageous when one is dealing with compounds which do not react, or react only poorly with the substances listed in 1–10. Colors of widely-varying intensities are obtained which fluoresce mostly in the UV (360 mμ region). Moisture interferes!

(16) *Antimony pentachloride* for various classes of steroids, like sterols [205, 264, 265], estrogens [199, 200, 266], steroid amines [194], and others [231, 249]: 5–50% solution in chloroform, dip or spray, then heat briefly if necessary (moisture-sensitive).

(17) *Zinc chloride* for various steroids:

(a) 30% in absolute methanol, spray and heat to 130° for periods up to one hour [325].

(b) Spray with a solution of 20 g anhydrous zinc chloride in 30 ml glacial acetic acid, heat to 90°. The colors or fluorescences thus obtained can sometimes be appreciably strengthened by subsequent treatment with a solution of benzoyl chloride–chloroform (1:1) and drying [267]. See also [266].

(18) *Tin tetrachloride* [139]: 30% in chloroform. Dip, dry 20 min at 90°. With certain steroids this procedure gives good fluorescence in UV-light.

(19) *Iodine–potassium iodide* gives a blue color with cortisone and a few other steroid compounds [233, 266, 268]: 0.3% iodine in 5% aqueous potassium iodide. Subsequent treatment of the (sprayed) chromatograms with ether is said to make the reaction much more sensitive, especially for oxygenated progesterone derivatives [269].

(20) *Iodine* [270] in the form of vapors or dissolved in petroleum ether also gives fugitive yellow to brown spots, mostly of moderate intensity, with various steroids [177, 249, 271], bile acids [240]. This reagent can also be used in combination with periodic acid [231].

(21) *Ferric chloride* or *ferric chloride–potassium ferricyanide* for estrogens or enols (blue spots): 2% ferric chloride in absolute methanol [266] or, much better, a fresh mixture of equal parts 1% aqueous ferric chloride and 1% aqueous potassium ferricyanide [272].

(22) *Aromatic aldehydes + acids* for various steroids, especially glycosides and aglycones, *e.g.*:

(a) Anisaldehyde–sulfuric acid–glacial acetic acid [213, 260, 273], additional references in [225].

(b) Dimethylaminobenzaldehyde–sulfuric acid–acetic anhydride [260, 271].

(c) Cinnamic aldehyde–sulfuric acid–acetic anhydride–antimony trichloride [275] for saponins.

(d) Furfurol–glacial acetic or phosphoric acid [225, 276].

(e) Vanillin–phosphoric acid [274, 276, 277] for 17-hydroxy-20-keto-20-methyl-steroids and others.

(f) *p*-Hydroxybenzaldehyde–ethanol–sulfuric acid for sapogenins [382, 383].

(23) *3,5-Dinitrobenzoic acid* (Kedde-reagent) for cardenolides (blue-violet spots):

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Spray with 1% in 0.5 *N* KOH in methanol–water 1:1 [208] or with a mixture of equal volumes of 2% solution in methanol and 2 *N* KOH [248].

(24) Potassium iodobismuthate (Kraut-Dragendorff-reagent) and potassium iodoplatinate for Girard-derivatives [190] and steroid alkaloids [194, 278, 279].

(25) If the reagents mentioned above do not permit the identification of the steroids to be analysed on the chromatogram, there are other possibilities, as indicated in the following compilation:

Reagent	*Test for*
Salicyloylhydrazide (+ indoaniline test [381])	steroids with reactive oxo-groups
Conc. or 90% H_2SO_4 [122, 268, 280]	various steroids
Sulfuric acid–glacial acetic acid [213, 263]	various steroids
15% fuming sulfuric acid [266]	estrogens
Phenolsulfonic acid–phosphoric acid [266]	estrogens
Osmium tetroxide [281]	α,β-unsaturated ketones
Silver nitrate, exposure to light and developing [198]	5,6-dibromides
Phthalic acid–zinc chloride [282]	estrogens
p-Phenylenediamine–phthalate [302]	Δ^4-3-ketosteroids
Millon's reagent [266]	estrogens
Bromine water [266]	estrogens
Brown nitric oxide vapors–ammonia [284]	estrogens
Folin-Ciocalteu-reagent [190, 285]	estrogens, reducing steroids
Diazo-reagent (Pauly) [255, 294]	phenolic steroids, estrogens
Sodium nitroprusside (Legal-reagent) [228, 267, 273]	cardenolides
Picric acid (Baljet-reagent) [228, 249]	cardenolides
p-Nitroaniline [276]	cardenolides
Iodate–benzidine [283]	glycosides
Permanganate [233]	various steroids
Permanganate–periodate [286]	various steroids, saponins
Fluorescein (0.0005%, contrast procedure) [271]	various steroids
Eriochromecyanin [198]	strongly lipophilic sterols
Mayer's reagent ($HgCl_2$, KI, $ZnCl_2$) [278]	steroid alkaloids
Fröhde's reagent (Mo-H_2SO_4) [278]	steroid alkaloids
Blood (biological assay) [247]	sapogenins

Photocopies or photographs of spots fluorescing in UV-light can be prepared by using suitable filters [287, 288] in a manner analagous to the preparation of photocopies of substances absorbing in the UV.

(26) *Radioautography*. Radioactive steroids or steroid derivatives can be determined directly on the chromatogram with a counter [155, 289] or made visible by contact with X-ray film for a period of 1 day to several weeks [290, 291, 292, 293].

In the simplest case, the examination of a paper chromatogram provides 2 different pieces of information, which, combined, constitute a limited characterization or a preliminary identification of the substance:

1. R_F-value $= \left(\frac{\text{distance from starting point to the center of the spot}}{\text{distance from starting point to solvent front}}\right)$

2. Color reaction or physical property.

Various factors diminish the value of these data, however, particularly when one considers the large number of similar steroids:

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With respect to 1: Despite great care in carrying out the chromatography and in holding all the conditions constant (temperature, saturation, quantities of the phases, time of development, etc.) the R_F-values are subject to certain variations. It is therefore preferable to compare the speed of migration of an unknown substance to that of a standard substance S which migrates with about the same speed, instead of to that of the solvent front F. The R_S-value

$$\left(\frac{\text{distance from starting point to the center of the spot}}{\text{distance from starting point to the substance } S}\right)$$

so obtained is much more constant, except in cases where displacement effects occur, or where two substances run very close together and have very small or very large R_S-values. Moreover the R_S-value can be given even when the solvent front has long since run off the paper, as it must often be allowed to do if one is to develop the chromatogram long enough to obtain a good separation. It is best to choose for S a substance (steroid, dye, etc.) which migrates to the center part of the chromatogram under the given conditions (*cf. e.g.* [150, 295]). For a good chromatographic characterization one should take care that the substances to be analysed do not run too close to the start, or to the solvent front. In the first case, one must run the chromatogram longer, or use a more polar solvent; in the second, one must use a less polar solvent. Even under these conditions, and considering that the length of the spot is 1.5–3 cm, it is necessary to reckon with a certain scatter of the R_S-values over a fairly long period. When single substances or especially several components of a mixture are to be characterized or identified by paper chromatography, one always chromatographs suitable reference substances at the same time. Furthermore, considerably more certain results are obtained by using various solvent systems. At this point we may recall several factors which can affect the rate of migration, such as irregular impregnation, incomplete saturation of the vessels, major variations in temperature, the height to which the troughs are filled, the manner of hanging up the paper in the trough (capillary action), duration of the chromatography. When exact comparisons are to be made, the outermost edges of the sheet or the two outer strips in a series should be left blank, since in this region the mobile phase of certain systems often runs somewhat behind the solvent front of the main portion.

With respect to 2: The nature and intensity of the color reactions on chromatograms are likewise subject to certain variations. These may depend upon the concentration of the substances, impurities or superimposition (mixed colors), method of carrying out the color reaction (*e.g.* strength of the spray, period of heating, moisture, impurities), or upon residues of solvents (*e.g.* formamide). These considerations apply particularly to color reactions which evoke fluorescence in UV-light. One should make it a rule wherever possible to call upon more than one color reaction for characterization (parallel chromatograms).

By combining two or more R_F- or R_S-values *and* color reactions, most steroids can be characterized very well indeed, and identified with comparison substances with a very high degree of probability (*cf.* 150, 295]). In view of the large number of

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isomeric steroids, however, the possibility should never be overlooked that two different compounds are occasionally indistinguishable by paper chromatography. For this reason, the performance of mixed chromatography, in analogy to mixed

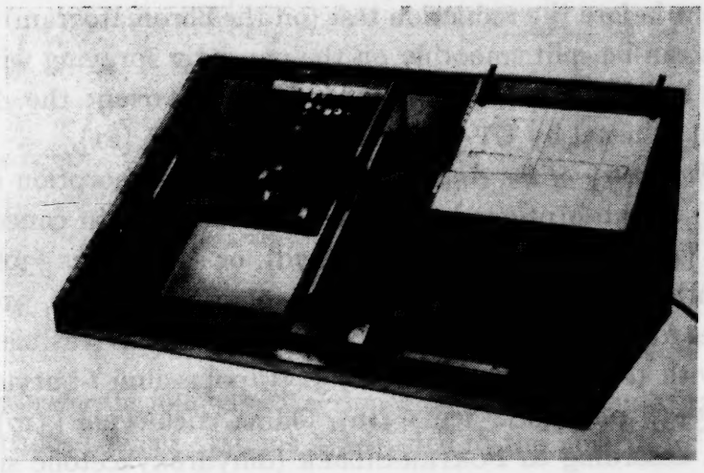

Fig. 12. Pantograph for the evaluation of photocopies and chromatograms.

melting points, has little significance. For the preparation of permanent records it is useful to enter the R_F- or R_S-values and color reactions, and also the UV-absorption, if any, on suitable forms; an R_F-measuring device or pantograph (Fig. 12 [218]) is helpful.

(f) Additional methods of identification for use in connection with chromatography

(1) *Chromatography of derivatives.* A very useful technique is the comparative chromatography of free steroids and their acetates [178, 280] or their oxidation products after treatment with chromic acid [178, 180, 280, 293] or periodic acid [296]; this is carried out on a micro-scale, sometimes with the aid of the isotope dilution technique [293]. From the differences between the R_F-values of the free and altered steroids (quantities of 0.01–100 μg are necessary), or from the very fact that a reaction has or has not occurred, it is often possible to draw important conclusions about the number and position of hydroxy groups (primary, secondary, tertiary; *cf.* also Section 3, p. 184). Difficultly separable saturated and unconjugated unsaturated 17-ketosteroids can be differentiated by treatment with perbenzoic acid, since the unsaturated compounds are converted to more polar derivatives, and the saturated ones remain unchanged [211]. An additional identification of 17-ketosteroids by chromatography of their Zimmermann-colorcomplexes has been proposed [297].

(2) *Microchemical reactions on the chromatogram.* Under certain circumstances the steroids can be oxidized, reduced, or hydrolyzed on the chromatogram itself, and the reaction products then identified by color reactions, or spectrographically. By alternate oxidation, reduction, and color tests the nature of the side chain of C_{21}-steroids can be determined to a certain extent [298], although side reactions must be

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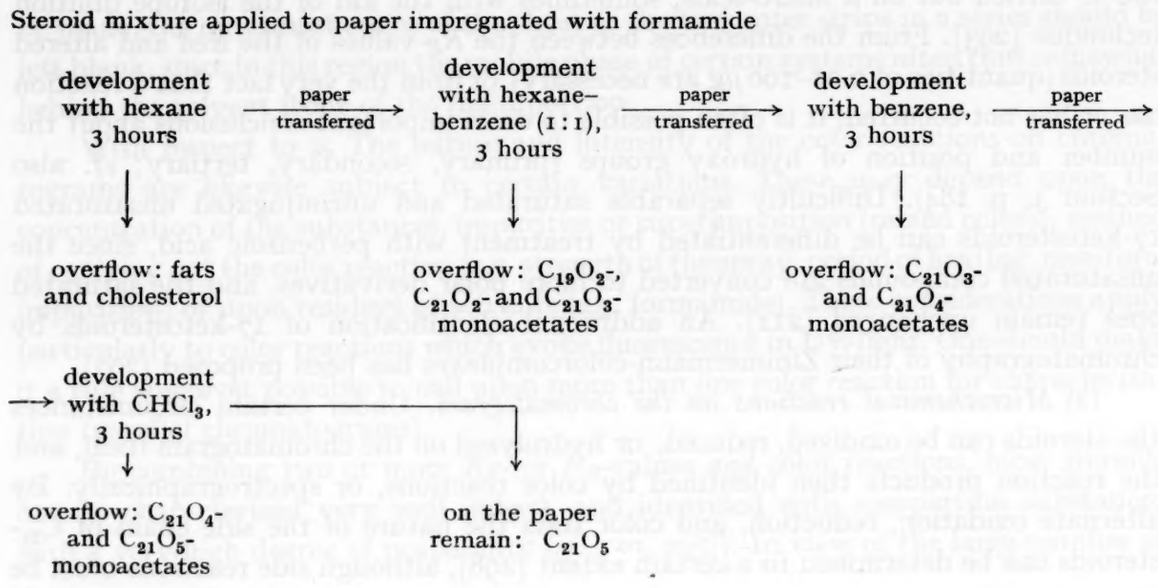
taken into account. Alkaline oxidation with periodic acid, followed by a positive DNB-reaction appears to be specific for a glycerol side chain [299], while all 17,20-diols react with acid periodate [300]. It is said to be possible to distinguish between dihydroxyacetone and ketol side chains on the basis of the greater sensitivity of the former to alkali-treatment before the reduction test (on the chromatogram) [301]. Ketals of Δ^4-3-ketosteroids can be split smoothly on the paper by spraying with 50% aqueous glycolic acid and heating 20 minutes at 90° in a drying oven; the Δ^4-3-ketones can then be identified as usual by UV-absorption (photocopy) [81].

(3) *Spectrophotometry of the eluates.* Besides the UV-absorption of the eluates in alcohol (*cf.* [311]) the absorptions in alkaline ethanol [303], in concentrated sulfuric acid for steroids [178, 343, 344], estrogens [179], or sapogenins [306, 307], in phosphoric acid–sulfuric acid [308] or in 100% phosphoric acid [309, 310] provide additional possibilities for characterization. Of the color reactions feasible *in vitro*, we may mention those with tetrazolium blue [312, 313], potassium *t*-butylate ("soda fluorescence") [314, 315], perchloric acid [316], iodine trichloride [317], and especially phenylhydrazine according to PORTER-SILBER (dihydroxyacetone side chain [318]). Periodic acid oxidation may also be called upon for characterization of the side chain, *e.g.*, by the evolution of microquantities of formaldehyde, and also for characterization of the remainder of the molecule (*e.g.*, formation of 17-ketones, of acids or of 5-membered lactone rings from aldosterone).

(g) *General separation schemes*

For many purposes it suffices to run chromatograms in a single system, or parallel chromatograms in several systems. With very complicated mixtures like extracts of urine or of organs, for example, a complete separation is possible only by repeated chromatographing, fractional elution and rechromatographing in different systems. One proceeds for example, as in the following scheme [178]:



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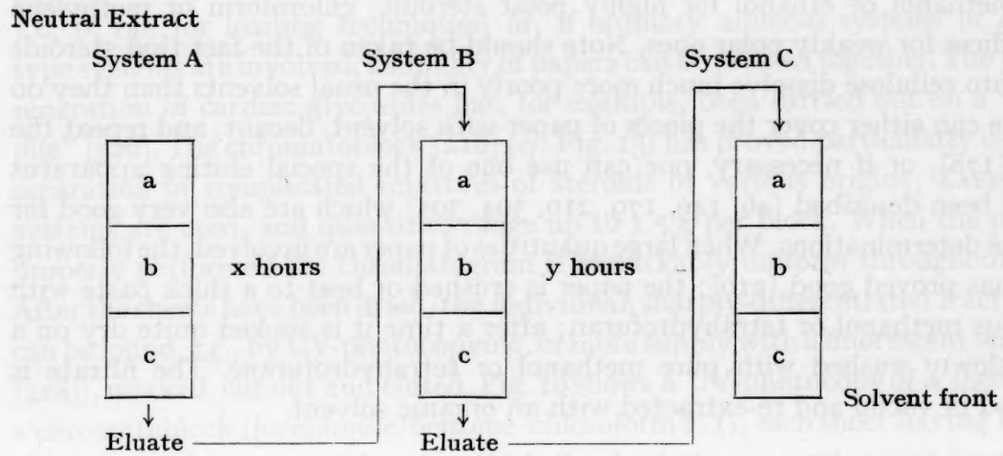
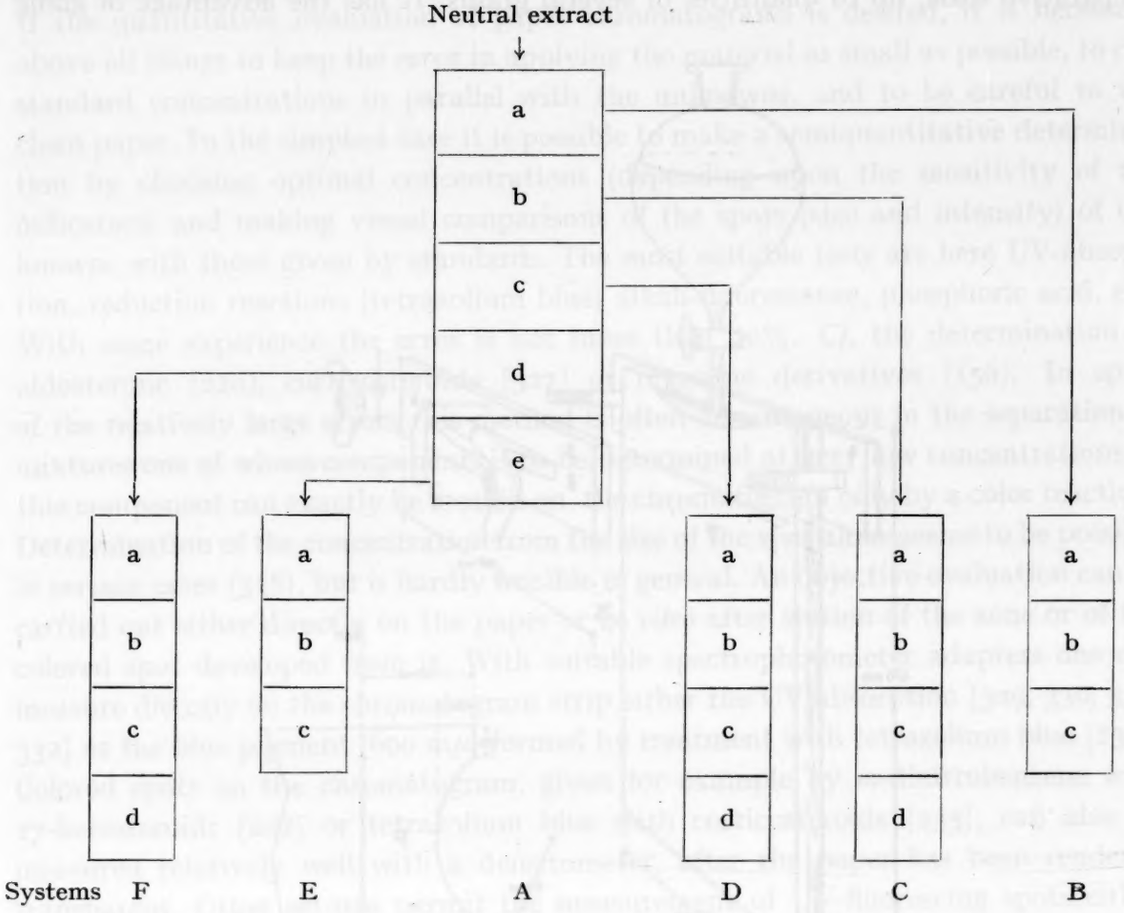
or in a similar manner [320, 321, 322]:



We generally prefer the following fractionation (*cf.* for example [323]), in which the polarity of the various solvent systems is adjusted to the polarity of the eluate from the first chromatogram.



If necessary, re-elute the fractions from B–F and rechromatograph in other systems.

In this connection we should mention briefly the possibility of eluting the steroids from the chromatograms. For the direct elution for example, of bands or strips cut

transverse to the direction of development of the chromatogram, it is best to use 80–100% methanol or ethanol for highly polar steroids; chloroform or methylene chloride suffices for weakly polar ones. Note should be taken of the fact that steroids adsorbed onto cellulose dissolve much more poorly in the usual solvents than they do *in vitro*. One can either cover the pieces of paper with solvent, decant, and repeat the procedure [178], or if necessary, one can use one of the special eluting apparatus which have been described [46, 149, 179, 210, 304, 305] which are also very good for quantitative determinations. When large quantities of paper are involved, the following procedure has proved good [210]: the paper is crushed or beat to a thick paste with 20% aqueous methanol or tetrahydrofuran; after a time it is sucked quite dry on a filter and slowly washed with pure methanol or tetrahydrofurane. The filtrate is concentrated in vacuo and re-extracted with an organic solvent.

(*h*) *Preparative paper chromatography*

In recent years paper chromatography has been applied more and more even on a preparative scale, up to quantities of several grams. It has the advantage of going

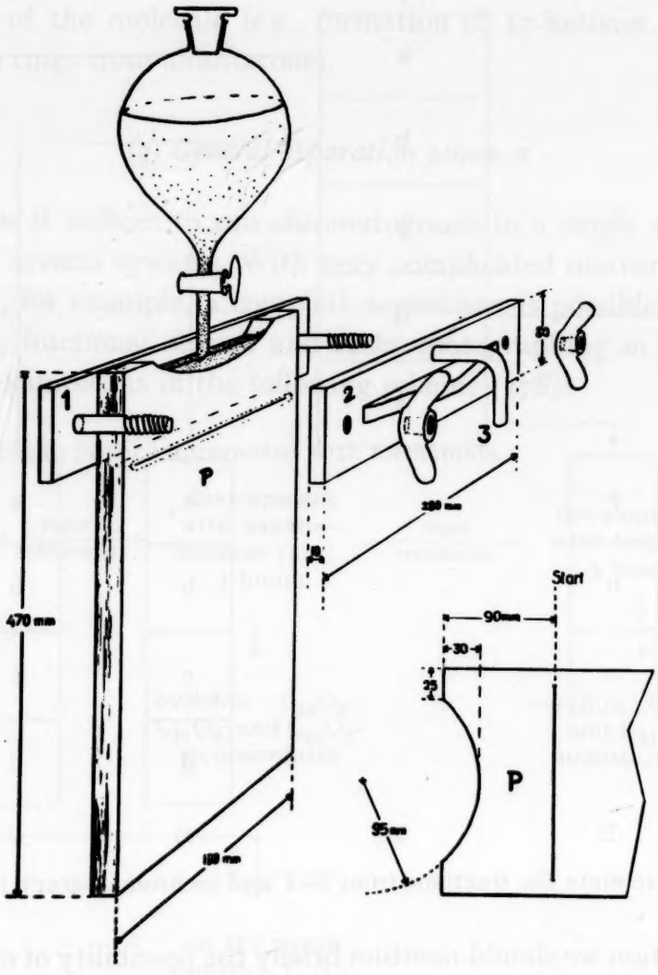


Fig. 13. Chromatoblock for preparative paper chromatography.

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rapidly and yielding only relatively few, well defined fractions. One can use a large number of individual sheets [46] (in a space-saving, slotted trough ([218]; *cf.* Section 1 c, p. 148 for loading techniques) or, if ordinary aqueous systems or ZAFFARONI-type systems are involved, a number of papers can be pressed together. The preparative separation of cardiac glycosides has, for example, been carried out on a "chromatopile" [326]. The chromatoblock [218] (*cf.* Fig. 13) has proved particularly useful for the separation of complicated mixtures of steroids of various origins; ZAFFARONI-type systems are used, and quantities range up to 1.5 g per block. When the procedure is properly performed the chromatogram is remarkably uniform throughout the block. After the sheets have been dried, the individual, sharply-differentiated fractions (zones) can be found, *e.g.*, by UV-photocopying, or more simply with a fluorescent screen(Fig. 11 [218]), marked, cut out and eluted. Fig. 10 shows a UV-photocopy of a sheet from such a chromatoblock (formamide/benzene–chloroform 1:1), each sheet having been loaded with 10 mg of an adrenal fraction. With this load, the separation is at least as good as in an analytical chromatogram.

(*i*) *Quantitative methods*

If the quantitative evaluation of paper chromatograms is desired, it is necessary above all things to keep the error in applying the material as small as possible, to run standard concentrations in parallel with the unknowns, and to be careful to use clean paper. In the simplest case it is possible to make a semiquantitative determination by choosing optimal concentrations (depending upon the sensitivity of the indicators) and making visual comparisons of the spots (size and intensity) of unknowns with those given by standards. The most suitable tests are here UV-absorption, reduction reactions (tetrazolium blue) alkali-fluorescence, phosphoric acid, etc. With some experience the error is not more than 20%. *Cf.* the determination of aldosterone [210], corticosteroids [327] or pregnene derivatives [150]. In spite of the relatively large errors this method is often advantageous in the separation of mixtures one of whose components is to be determined at very low concentrations, if this component can exactly be located on the chromatogram only by a color reaction. Determination of the concentration from the size of the spot alone seems to be possible in certain cases [328], but is hardly feasible in general. An objective evaluation can be carried out either directly on the paper or *in vitro* after elution of the zone or of the colored spot developed from it. With suitable spectrophotometer adapters one can measure directly on the chromatogram strip either the UV-absorption [329, 330, 331, 332] or the blue pigment (600 mμ) formed by treatment with tetrazolium blue [133]. Colored spots on the chromatogram, given for example by *m*-dinitrobenzene with 17-ketosteroids [227] or tetrazolium blue with corticosteroids [235], can also be measured relatively well with a densitometer, after the paper has been rendered transparent. Other set-ups permit the measurement of UV-fluorescing spots either directly on the paper (*e.g.*, alkali-fluorescence of corticosteroids [237] or by densitometry of fluorescence photographs, in the case of cardiac glycosides [287] after treatment with trichloroacetic acid.

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More exact results are generally obtained when one elutes the substances (located if necessary on parallel chromatograms) as such, and measures spectrophotometrically *in vitro*, either directly, or after treatment with reagents; the eluate from a chromatogram to which no substance has been applied is measured as a blank (care should be taken to obtain low blanks by washing the papers [212]). The adsorption at 239–240 mμ is often used for example for Δ^4-3-ketosteroids (corticosteroids, etc. [150, 178, 223, 280, 304, 305]) at 510–525 mμ after formazan formation with tetrazolium blue (for corticosteroids [178, 305]) or at 526 mμ after reaction with xanthydrol (for cardenolides [248]). Other reactions may be used as desired, for example the ZIMMERMANN-reaction for 17-ketosteroids [211], LIEBERMANN-BURCHARD for cholesterol [196], concentrated or fuming sulfuric acid for various steroids [321, 212], alkali-fluorescence for Δ^4-3-ketosteroids [315], reaction with hydroxylamine and ferric chloride for the determination of acetyl groups [178] or treatment with other reagents mentioned in Section 1 f, p. 159.

It is also possible to carry out the color reaction on the chromatogram and measure the pigment formed *in vitro* after elution. For example, formazan spots can be eluted from paper with pyridine-hydrochloric acid, or better, with tetrahydrofurane [334, 335] or with ethyl acetate–methanol (7:3) [336] (absorption maximum 550–560 mμ); the blue product formed from reducing steroids and arsenomolybdate can be eluted merely with water [236] (absorption maximum 650–660 mμ). These methods have the advantage that the substance to be determined (or the colored reaction product) can be cut out of the chromatogram very precisely, although the color-formation on the paper and the elution of the colored substances contain certain sources of error. In the opposite case, where the untreated steroid is eluted first, and then caused to react *in vitro*, the color formation is better controlled, to be sure, but the exact location of the substance to be eluted presents difficulties, unless it can be done with the aid of UV-absorption, fluorescence, or radioactivity (caution against losses caused by UV-irradiation [220, 221]). Finally, mention should be made of the very promising quantitative determination of radioactive steroids or steroid derivatives on the paper or *in vitro*. This method is applicable down to very low concentrations, and can in certain cases (double labeling) eliminate all losses [289–293]).

2. Special methods

A general survey of the applicability of selected systems to various classes of steroids has been given in Fig. 4 (p. 144); in these cases the chromatogram was developed only until the solvent front had reached the bottom edge of the paper (2.5–3 hours). The substances in the upper part of the chromatogram can be displaced downward at will by extending the time by a factor of 2 or more. This may serve as a criterion for determining the proper time for chromatographing substances whose R_S-values are given in any of the following tables, S being corticosterone or cortisol, for example. Depending on the R_S-value, one develops the chromatogram until S has migrated to some point between the upper and lower quarters of the paper.

References p. 186.

As has been mentioned above, it is customary, in view of the large number of compounds of similar polarity, to make identifications, even within a single class, not only on the basis of R_F- or R_S-values, but also on the basis of various indications which are specific for certain functional groups, like UV-absorption (α,β-unsaturated ketones), tetrazolium blue–alkali (reducing side chains or cyclic ketols and Δ^4-3-ketones), ZIMMERMANN-reaction (principally 17-ketones), dinitrophenylhydrazine (reactive ketones) etc. By these means even compounds which chromatograph with equal velocities can be differentiated, and at the same time some inkling can be obtained as to the possible constitution of unknown spots. As an example, we may contrast the behavior of the nowadays very important 1:4 diene-3-ketosteroids with the Δ^4-3-ketosteroids. Both are easily detectable by UV-photocopy, but the yellow alkali-fluorescence appears only with the latter compounds.

For these reasons, the steroids entered in the tables in the following sections are arranged not only according to R_F- or R_S-values, but are frequently subdivided *e.g.* under the headings UV + BT +, UV + BT —, UV — BT +, UV — BT —, or by giving the UV-absorption in combination with DNB or DNPH * reactions. However, we have not attempted to quote all of the R_S- and R_F-values and all the qualitative and quantitative data on the color reactions which may be found in the literature, because the conditions under which the experiments are performed vary considerably from place to place, depending upon the experience of the workers, so that it is always necessary to chromatograph standards of comparison along with the unknowns in order to achieve positive identifications. The tables which follow are intended primarily to provide a thorough orientation by giving numerous examples of all types of steroids; in this connection, see also the compilation in [150].

All values refer to room temperature (usually 22° C) when nothing is stated to the contrary, and are averages of several runs, at least in the case of the author's own work.

(*a*) *Sterols*

The original ZAFFARONI-systems are too polar for most sterols, but a system with phenyl cellosolve (ethylene glycol monophenyl ether) as the stationary phase and heptane, for example, as the mobile one, on Whatman No. 7 has proved generally applicable [267, 380]. The *R*-values for various derivatives are given in Table 30, see [264]. Antimony trichloride and antimony pentachloride [205, 265] serve as test reagents; the former gives sensitive color reactions [267] especially with Δ^5-3β-hydroxysteroids. *Cf.* [291] for the chromatography of cholesterol containing ^{14}C. Reversed phase chromatography is frequently used for sterols. Although it is very difficult to make the paper hydrophobic with quilon [205], it can be done quite well

* The abbreviations used here and in the tables are as follows: UV = UV-absorption at 240 mμ; BT = reduction with tetrazolium blue; DNB = alkaline *m*-dinitrobenzene; DNPH = dinitrophenylhydrazine; P = propylene glycol; F = formamide; Tol = toluene; Be = benzene; Cy = cyclohexane; A, B_{1-5}, BL_1, C and E_2B denote systems of the BUSH-type (for their compositions see Fig. 4, p. 144); DH = dihydro; TH = tetrahydro.

References p. 186.

TABLE 30

STEROLS IN THE SYSTEM PHENYL CELLOSOLVE/HEPTANE [264]

	R_S	$SbCl_3$ in $CHCl_3$ (20%)
Monohydroxysterols	S = cholesterol, 5-h development	
C_{27}-Sterols		
Cholesterol	1.0	rose
Lathosterol	0.9	reddish-violet
7-Dehydrocholesterol (2F)	0.7	blue green
Cholestanol	1.1	rose violet
Coprosterol	1.3	rose
C_{28}-Sterols		
Brassicasterol (2F)	1.0	rose
Ergosterol (3F)	0.6	blue green
C_{29}-Sterols		
β-Sitosterol	1.0	rose
α-Spinasterol (2F)	0.9	grey
Stigmasterol (2F)	1.0	rose
Di- and trihydroxysterols	S = cholest-5-en-3β,7β-diol, 20-hr development	
Cholest-5-en-3β-7β-diol	1.0	blue green
Cholest-5-en-3β,7α-diol	0.7	blue green
Cholest-5-en-3β,24-diol	0.4	brown
Cholest-5-en-3β,25-diol	0.4	brown
Cholest-4-en-3β,6-diol	0.6	light brown
Cholestan-3β,5α,6β-triol	0.5	yellow brown

The Δ^7-sterols and 7α- or 7β-hydroxy-derivatives give brilliant colors immediately on being sprayed; others give the colors only after brief heating to 100° C, stanols only on prolonged heating.

with paraffin oil, using glacial acetic acid–chloroform–paraffin oil mixtures (65:25:10) as the mobile phase [196, 197]; ascending, Whatman No. 3).

The R_F-values are for

Cholesterol	0.62
Cholesterol acetate	0.34
Cholesterol caproate	0.16
Cholesterol stearate and palmitate	0.02
Cholesterol oleate	0.03

These compounds were also determined semi-quantitatively after elution. Vitamin D and a number of related substances have been chromatographed with a similar system [193]; paper made hydrophobic with 5% paraffin oil in light petrol; mobile phase:

(a) Ethyl cellosolve–*n*-propanol–methanol–water (35:10:30:25).

(b) *n*-Propanol–methanol–water (15:82:3).

(c) 95% methanol; ascending, Whatman No. 2.

Test reagent: antimony trichloride. For the application of paper impregnated with aluminum soap, *cf.* [204], and for an attempt at a two-dimensional separation, [192], silicic acid paper (adsorption) being used in the first direction and hydrophobic paper (vaseline; partition) in the second direction. For recent work on this class of compounds, see [384, 387].

References p. 186.

(*b*) C_{18}-*Steroids* (*estrogens*)

Estrogens can be chromatographed with no particular difficulty in the usual systems (*cf.* Table 31, systems I, II, III). *Cf.* also Table III in [150]. It is better to use a more polar system for estriol than for estradiol and estrone. All are easily detectable, especially with ferric chloride–potassium ferricyanide, Folin-Ciocalteu, antimony pentachloride, antimony trichloride, phosphoric acid, *m*-dinitrobenzene for estrone, diazo-reagent, and others (*cf.* the test reactions, Section 1 e, p. 151). The separation of a series of estrogens with the systems P/methylcyclohexane, F/*o*-dichlorobenzene, F/cyclohexane and F/methylene chloride has been described [179, 266]. One or more runs permitted the separation of estriol, estradiol-17α and -17β, estrone, 6-dehydroestrone, 7-ketoestrone, equilin, equilenin, 17α-ethynylestradiol, etc. Various color reactions are described and a quantitative determination by spectrophotometry of the eluates at 280–282 mμ is given. With the system benzene–methanol–water (10:8:2) one obtains at 24° the R_F-values given in Table 31 (system V) [282]. Identification is made with phthalic acid–zinc chloride (yellow green fluorescence). Still other variations have been described for chromatography in neutral systems [285, 337], and more recently in [379, 388]. There is a lowering of the R_F-values when the stationary phase is an aqueous solution of lithium chloride or calcium chloride instead of water (mobile phase toluene–methanol [294, 338]), Table 31, system VI.

In the case of the estrogens, many variations are made possible by the use of alkaline phases: the Whatman No. 1 paper is first equilibrated with both phases (stationary 0.5 *N* NH_4OH, mobile chloroform–benzene–*N* NH_4OH (1:9:1)) and the descending chromatogram is then developed for 2–3 hours [255]. For R_F-values see

TABLE 31

PAPER CHROMATOGRAPHY OF ESTROGENS

	System							
	I	*II*	*III*	*IV*	*V*	*VI*	*VII*	*VIII*
Estriol	0.00	0.00	0.05	0.05	0.18	0.00	0.02	
Estradiol-17β	0.36	0.93	1.40	1.29	0.68	0.27	0.78	
Estradiol-17α						0.45		
Estrone	2.22	1.62	2.01	1.57	0.86	0.78	0.89	
Equilin						0.65		
17α-Ethynylestradiol						0.52		
Estradiol-17β diacetate								0.42
Estradiol-17β dipropionate								0.20
Estradiol-17β dibenzoate								0.68
Estron acetate								0.72

I = propylene glycol/toluene
II = formamide/benzene–$CHCl_3$ (1:1)
III = petroleum ether–benzene–methanol–water (3:7:5:5) = BL_1
IV = BL_1, 38° C
} R_S-values (S = corticosterone)

V = benzene–methanol–water (10:8:2)
VI = toluene–methanol–LiCl or $CaCl_2 \cdot 2H_2O$ in water
VII = $CHCl_3$–benzene–*N* NH_4OH (1:9:1)
VIII = silicone paper, polar phase of water–methanol–benzene–petroleum ether (2:7:3:6)
} R_F-values

References p. 186.

Table 31, system VII. For similar alkaline systems, *cf.* [284, 339]. Reversed phase chromatography has also been used for estrogens, especially in the form of their esters or ethers [199, 200]. The paper is made hydrophobic with 5% silicone (Aquatex 4040) in cyclohexane, and dried for 1 hour at 110° C. The bottom layer of a mixture of water, methanol, benzene, petroleum ether (2:7:3:6) is used as the mobile phase, and the upper layer as the stationary, with which the vessel is saturated and equilibrated: R_F-values, Table 31, system VIII. Finally, estrogens in the form of their azo-derivatives can be separated in BUSH-type systems [238, 340]; this procedure is, however, probably no longer so important in comparison to the more modern methods.

(c) C_{19}-*Steroids* (*17-ketosteroids*)

The R_S-values of numerous C_{19}-steroids in various systems are given in Tables 32–35, [36]; *cf.* also Table III in [150]. These C_{19}-steroids are conveniently divided into groups which are detected in various ways (none by reduction) *e.g.* Δ^4-3-ketosteroids (UV-absorption at 240 mμ, alkali fluorescence), 17-ketosteroids (DNB), steroids without reactive keto groups (antimony trichloride, phosphoric acid, phosphomolybdic acid, etc.). The latter group is usually the most difficult to detect, especially the 11-keto compounds, whereas triols react relatively well with antimony trichloride. A very

TABLE 32

R_S-VALUES (S = CORTEXONE) OF C_{19}-STEROIDS (UV +) [36]

	P/Tol	*F/Be–Cy (1:1)*	B_3	A^{390}	*DNB*	*NaOH-fluorescence*
11β-Hydroxyandrost-4-en-3,17-dione	0.60	0.32	0.32		+	+
Androsterone	1.07	0.89	0.75	0.45	+	+
Androst-4-en-3,17-dione	1.18	1.71	1.49	1.86	+	(+)
Testosterone	0.64	1.04	0.79	1.17	o	+
17α-Testosterone (*epi*-testosterone)	0.74	1.23	0.93	1.47	o	+
Testololactone	0.93	0.42	0.41	0.19	o	+
1-Dehydro-testololactone	0.67	0.21	0.16		o	o

TABLE 33

R_S-VALUES (S = CORTEXONE) OF C_{19}-STEROIDS (UV —, DNB +) [36]

	P/Tol	*F/Be–Cy (1:1)*	B_3	A^{390}	*F/Be*	*DNPH*
3β,11β-5α-Androstan-17-one	0.14	0.20	0.08			(+)
3β-Hydroxyandrost-5-en-11,17-dione	0.50	0.45	0.32		0.76	(+)
3β-Hydroxy-5α-androstan-11,17-dione	0.50	0.44	0.34		0.75	(+)
3α-Hydroxy-5β-androstan-11,17-dione	0.65	0.66	0.47	0.35		(+)
3β-Hydroxy-5β-androstan-11,17-dione	0.67	0.75	0.50		0.91	(+)
3α-Hydroxy-5α-androstan-11,17-dione			0.59			(+)
5α-Androstan-3,11,17-trione	1.22	1.31	1.12		1.14	+
5β-Androstan-3,11,17-trione	1.22	1.33	1.14	1.11	1.15	+
Dehydro*epi*androsterone	0.84	1.51	0.84	1.94		(+)
Androsterone	1.01	1.79	1.35	3.12		(+)

References p. 186.

TABLE 34

R_S-VALUES (S = CORTICOSTERONE) OF C_{19}-STEROIDS (DNB +) [36]

	P/Tol	F/Be	F/Be–$CHCl_3$ (1:1)	$BL_1^{38°}$	DNPH
3β,11β-Dihydroxyandrost-5-en-17-one	0.60	1.50	1.16	1.06	(+)
3β,11β-Dihydroxy-5α-androstan-17-one	0.68	0.66	0.98	1.20	(+)
3α,11β-Dihydroxy-5β-androstan-17-one	0.74			1,20	(+)
3α-Hydroxy-5β-androstan-11,17-dione	2.30	3.30	1.79		(+)
3β-Hydroxyandrost-5-en-11,17-dione	2.34	3.20	1.67	1.40	(+)
3α-Hydroxy-5α-androstan-11,17-dione		3.20	1.68		(+)
3β-Hydroxy-5α-androstan-11,17-dione	2.39	3.23	1.69	1.40	(+)
11β-Hydroxyandrost-4-en-3,17-dione	2.42		1.42	1.54 (22°)	+, UV +
3β-Hydroxy-5β-androstan-11,17-dione	3.06	3.80	1.80	1.48	(+)
5α-Androstan-3,11,17-trione	5.57	4.78	1.92	1.67	+
5β-Androstan-3,11,17-trione	5.81	4.82	1.95	1.70	+

TABLE 35

R_S-VALUES (S = CORTICOSTERONE) OF C_{19}-STEROIDS (DNB —, UV —) [36]

	P/Tol	F/Be	F/Be–$CHCl_3$ (1:1)	$BL_1^{38°}$	DNPH
3β,11β,17β-Trihydroxyandrost-5-ene	0.06	0.29	0.31	0.36	o
5α-Androstan-3β,11β,17β-triol	0.05	0.28	0.34	0.42	o
5β-Androstan-3α,11β,17β-triol	0.06	0.35	0.39	0.51	o
5β-Androstan-3β,11β,17β-triol	0.11	0.50	0.47	tailing	o
5α-Androstan-3α,11β,17β-triol	0.12	0.60	0.50		o
3β,17β-Dihydroxyandrost-5-en-11-one	0.16	0.74	0.73	0.69	o
3β,17β-Dihydroxy-5α-androstan-11-one	0.15		0.70		o
3α,17β-Dihydroxy-5β-androstan-11-one	0.21	1.00	0.86	0.71	o
3β,17β-Dihydroxy-5β-androstan-11-one	0.24	1.00	0.86	0.78	o
3α,17β-Dihydroxy-5α-androstan-11-one	0.26	1.15	0.93		o
11β,17β-Dihydroxy-5α-androstan-3-one	0.79	1.45	1.20	0.99	+
11β,17β-Dihydroxy-5β-androstan-3-one	0.80	1.54	1.16	1.18	+

extensive study of the paper chromatography of C_{19}-steroids is due to SAVARD [180, 232], who preferred propylene glycol/ligroin as a solvent (50% propylene glycol in methanol for impregnation of Whatman No. 1).

For the determination of the "relative migration rate" (R_T) of the individual steroids, androsterone and 3α,11β-dihydroxy-5α-androstan-17-one (11-hydroxy-androsterone) are used as references; their absolute migration rates are approximately 1 cm/hour and 0.1 cm/hour. The R_T-value is calculated from the formulae

$$R_T = \frac{\text{cm migrated by substance x}}{\text{cm migrated by reference substance}} \cdot K$$

(K = cm/hour migrated by reference substance). The R_T-values of various groups of steroids can be summarized as in Table 36. Because of the high R_T-values of Group I substances in this system, they are better separated in a less polar solvent system. In Group II, the saturated 3,17- or 3,20-diketones are well separated from the corresponding Δ^4-unsaturated compounds. Isomers which differ only in their steric arrangement at C_5 are scarcely separable. This is true in part for Groups III and IV also (in this connection, see Section 3, p. 184). The analysis of complex mixtures of steroids can under some circumstances

References p. 186.

TABLE 36

R_T-VALUES OF VARIOUS GROUPS OF STEROIDS [180]
SOLVENT SYSTEM: PROPYLENE GLYCOL/LIGROIN

Group	*Steroid groups*	*R_T-Values*
I	Monoketosteroids, Monohydroxysteroids	3.0
II	Diketosteroids	1.5–3.0
III	Monohydroxy-monoketosteroids	0.2–1.5
IV	Monohydroxy-diketosteroids, Triketosteroids	0.2–1.5
V	Dihydroxy-monoketosteroids, Monohydroxy-triketosteroids, Dihydroxy-diketosteroids	0.01–0.2

be facilitated by separating the 3α- and 3β-hydroxysteroids beforehand with the aid of digitonin. Displacement effects have been observed with ketosteroids which migrate with almost the same speed. For further details, we refer the reader to the original literature.

If the paper strips which have been impregnated with propylene glycol are developed with benzene–cyclohexane (1:1) instead of with ligroin [230], the separation of epimeric 17-ketosteroids seems to be facilitated (*cf.* also Tables 33, 34). The same solvent system is used for separation and quantitative determination of 17-ketosteroids, the chromatogram being treated with alkaline *m*-dinitrobenzene until the color has developed optimally, drawn through an oil which renders it transparent, and measured directly with a densitometer [227]. Phenyl cellosolve heptane and propylene glycol heptane have also been used for the quantitative analysis of seven 17-ketosteroids from urine [211, 221]; the steroids are here eluted before being treated with the color reagent.

A further possibility for the separation of C_{19}- as well as C_{21}-steroids (relatively weakly polar) is offered by the use of the systems formamide/decalin or cyclohexane, propylene glycol/methylcyclohexane, butandiol/methylcyclohexane [231]. In following up this work, and with the additional use of formamide/methylene chloride, the qualitative identification of 12 $C_{19}O_3$-compounds, mostly 17-ketosteroids from urinary extracts, was achieved (see schema in [321]). For numerous color tests, see Section 1 e, p. 151.

As has been shown at the very beginning, BUSH-systems too, are of course suitable for the separation of C_{19}-steroids. Other similar examples are shown graphically [341], and also reported in [342]. In conclusion, mention should be made of a reversed phase procedure [201], using silicone-impregnated paper (5% Dow Corning Silicone No. 1107 in cyclohexane) and water–ethanol–chloroform (6:10:10) as the mobile phase (polar fraction), the vessel being equilibrated with the non-polar phase. The separations appear to be satisfactory, but no advantage whatsoever is to be noted, compared to the simpler systems mentioned above.

(*d*) C_{21}-*Steroids* (*corticosteroids*)

Along with the cardiac glycosides, this class of steroids has probably profited most from the recent (since 1950) introduction of suitable solvent systems [191]. From

References p. 186.

Tables 37–45 [36] one may see which solvent systems (ZAFFARONI- or BUSH-types) are suitable for the C_{21}-steroids, which vary widely in polarity, and also how these steroids can be distinguished by their *R*-values and test reactions. The foremost tests are here UV-absorption, trichloroacetic acid, reducing tests, sodium fluorescence, phosphoric acid, antimony trichloride, dinitrophenylhydrazine, and others (*cf.* Section I e, p. 151). By combination of selected solvent systems and tests, NEHER AND

TABLE 37

R_S-VALUES (S = CORTISOL) OF CORTICOSTEROIDS (UV +, BT +) [295]

	F/Be	*P/Tol*	*F/CHCl₃*	B_5	*C*	$C^{40°}$	$E_2B^{40°}$	H_3PO_4
17*α*,19-Dihydroxycortexone	0.13	0.19	0.36	0.20	0.31	0.35	0.64	red
6*β*,17*α*-Dihydroxycortexone	0.31	0.49	0.58	0.44	0.53	0.59	0.90	reddish-orange
6*β*-Hydroxycorticosterone	0.30	0.66	0.50	0.31	0.35	0.40	0.41	o
19-Hydroxycorticosterone	0.60	0.69	1.37	0.80	0.64	0.77	0.83	blue
Cortisol	1.00	1.00	1.00	1.00	1.00	1.00	1.00	yellow-green
6*β*-Hydroxy-11-dehydrocorticosterone		1.87	1.62	0.91	0.70	0.75	0.49	blue
3-Oxo-20,20,21-trihydroxypregn-4-en-18-oic acid 18-20 lactone		2.46	2.75	2.76	1.81	1.67	1.39	o
Cortisone	1.95	2.51	2.39	1.91	1.60	1.38	1.32	bluish
Aldosterone	1.18	2.59	2.27	1.52	1.03	0.97	0.73	o
19-Hydroxycortexone		4.11	2.79	2.34	1.71	1.40	1.24	o
Corticosterone			3.51		2.62			yellow-green
11*α*, 17*α*-Dihydroxycortexone (*epi*-Cortisol)		0.20	0.46	0.24	0.25	0.31	0.81	green
11*α*-Hydroxycortexone (*epi*-Corticosterone)		5.07	3.17	2.15	1.42	1.37	1.36	green

All these compounds give the typical yellow alkali fluorescence, except 17*α*,19-dihydroxycortexone (blue green), 19-hydroxycorticosterone (yellow-greenish) and 19-hydroxycortexone (blue green).

TABLE 38

R_S-VALUES (S = CORTISOL) OF CORTICOSTEROIDS (UV —, BT +) [295]

	P/Tol	*F/CHCl₃*	B_5	*C*	$C^{40°}$	$E_2B^{40°}$	H_3PO_4
Allo-tetrahydrocortisol (3*β*,5*α*)	0.20	0.22	0.33	0.42	0.57	1.12	violet-orange
Tetrahydrocortisol (3*α*,5*β*)	0.29	0.28	0.38	0.52	0.63	1.51	violet-orange
Allo-tetrahydrocortisol (3*α*,5*α*)	0.40	0.40	0.55	0.65	0.82	1.37	yellowish-lilac
Allo-tetrahydrocortisone (3*β*,5*α*)	0.40	0.79	0.70	0.60	0.77	1.78	light blue
Tetrahydrocortisone (3*α*,5*β*)	0.42	1.01	0.89	0.81	0.88	1.92	blue
3*β*,17*α*,21-Trihydroxy-5*α*-pregnan-20-one	2.34	2.50	2.87	2.02	1.63	2.51	red-violet
Allo-dihydrocortisol (5*α*)	2.67	1.61	1.86	1.58	1.43	1.40	orange-lilac
Dihydrocortisol (5*β*)	2.70	1.43	1.99	1.63	1.51	1.49	orange-lilac
3*α*,17*α*,21-Trihydroxy-5*β*-pregnan-20-one	2.80	2.79	2.81	1.99	1.81	2.71	o
Allo-tetrahydrocorticosterone (3*β*,5*α*)	4.00	2.63	2.64	1.87	1.67	1.61	(blue-violet)
Dihydrocortisone (5*β*)	5.31	2.84	2.83	2.04	1.73	1.66	blue
Tetrahydrocorticosterone (3*α*,5*β*)	5.43	2.60	3.24	2.21	2.01	1.62	o
Allo-dihydrocortisone (5*α*)	5.47	3.11	2.80	2.07	1.71	1.60	blue
Allo-tetrahydro-11-dehydrocorticosterone (3*β*,5*α*)	> 6.00	3.71	3.19	2.23	1.74	1.52	o
Tetrahydro-11-dehydrocorticosterone (3*α*,5*β*)	> 6.00	3.75	3.71	2.28	1.92	2.18	o

References p. 186.

TABLE 39

R_S-VALUES (S = CORTISOL) FOR CORTICOSTEROIDS (UV +, BT — AND UV —, BT —) [295]

	P/Tol	*F/CHCl₃*	B_5	*C*	$C^{40°}$	$E_2B^{40°}$	H_3PO_4
11β,17α,20β,21-Tetrahydroxypregn-4-en-3-one	0.25	0.48	0.20	0.23	0.32	0.68	yellow*
17α,20β,21-Trihydroxypregn-4-en-3,11-dione	0.84	1.48	0.67	0.50	0.58	0.79	o*
17α,20β,21-Trihydroxypregn-4-en-3,11-dione	0.95	1.59	0.72	0.46	0.58	0.72	o*
20β,21-Dihydroxypregn-4-en-3,11-dione	2.41	2.94	1.89	1.34	1.39	1.40	o*
5α-Pregnan-3β,11β,17α,20β,21-pentol	0.04	0.04		0.10	0.13	0.67	red**
5α-Pregnan-3β,17α,20β,21-tetrol	0.47	0.46		1.03	1.12	1.88	(reddish)**

* UV + ** UV —

TABLE 40

R_S-VALUES (S = CORTICOSTERONE) OF OXYGENATED CORTEXONE DERIVATIVES (MODERATELY POLAR CORTICOSTEROIDS, AND OTHERS) ALL UV +, BT + [36]

Hydroxycortexone	*P/Tol*	*F/Be*	*F/Be–CHCl₃ (1:1)*	BL_1	$BL_1^{38°}$	*F/Cy–Be (1:1)*	H_3PO_4
16α-	0.19	0.16	0.41		0.31		
15α-	0.23	0.19	0.45		0.27		
19-	0.28		0.49	0.40			
11α-	0.35		0.56	0.41			green
7?	0.40	0.26	0.51		0.47		
12α-	0.46	0.49	0.77		0.70		
18-	0.58		0.73	0.69		0.44	
6β-	0.59		0.73	0.69		0.49	
14α-	0.70	0.69	0.90		0.79		
17α-	0.77		0.98	1.06		0.99	red orange
8- or 9-	0.90	0.64	0.86		0.77		
11β- (= Corticosterone)	1.00	1.00	1.00	1.00	1.00	1.00	yellow green
11-Dehydrocorticosterone	2.30		1.36	1.42			(faint blue)
Cortexone	4.00		1.68	2.19	1.69		(orange)

All show the typical NaOH-fluorescence, except 19-hydroxycortexone (blue green) and 7α-hydroxycortexone (negative). See Table 2 in [295] for comparative R_S-values (S = corticosterone acetate) of the acetates of corticosteroids.

WETTSTEIN [295] succeeded in making paper chromatographic identification on a micro-scale of all the steroids which had up to that time been discovered in the adrenals; in this connection *cf.* Tables 37–45 and the graphical representation in [295]. Nearly all the solvent systems mentioned in Fig. 4 were used for this task.

REINEKE [150] has given R_S-values for a very large number of C_{21}-steroids with and without hydroxyl groups at C_{21}; reproduction of these data would lead us too far afield, but some of these steroids are listed in the tables in this chapter. Mention should be made, however, of the less-well known but equally excellent solvent systems [150, 378] carbitol (diethylene glycol monoethyl ether)/methylcyclohexane and carbitol–formamide (1:1)/hexane.

We wish also to note particulary the very useful data of ZAFFARONI [178, 233],

References p. 186.

TABLE 41

R_S-VALUES (S = CORTICOSTERONE) OF CORTICOSTEROIDS (UV — BT, +) [36]

	P/Tol	F/Be–$CHCl_3$ (1:1)	BL_1	H_3PO_4
3β,17α,21-Trihydroxy-5α-pregnan-20-one	0.14	0.50	0.64	red violet
3α,17α,21-Trihydroxy-5β-pregnan-20-one	0.18	0.53	0.32	(orange)
Allo-tetrahydrocorticosterone (3β,5α)	0.24	0.48	0.41	(blue violet)
Tetrahydrocorticosterone (3α,5β)	0.32	0.63	0.77 (38°)	o
Dihydrocortisone (5β)	0.30	0.68	0.26	blue
Allo-dihydrocortisone (5α)	0.32	0.46	0.31	blue
Allo-tetrahydro-11-dehydrocorticosterone (3β,5α)	0.48	0.83	0.86	o
Tetrahydro-11-dehydrocorticosterone (3α,5β)	0.60	0.97	0.71	o
Allo-dihydrocorticosterone (5α)	1.81 (tailing)	1.35	1.43	o
17α,21-Dihydroxy-5β-pregnan-3,20-dione	1.57 (tailing)	1.34	1.46	(orange)
Allo-tetrahydrocortexone (3β,5α)	2.03	1.76	2.03	o
Dihydro-11-dehydrocorticosterone (3α,5β)	2.81	1.68	1.73	o
Allo-dihydro-11-dehydrocorticosterone (3β,5α)	3.02	1.62	1.69	o

TABLE 42

R_S-VALUES (S = CORTICOSTERONE) OF CORTICOSTEROIDS (UV +, BT — AND UV —, BT —) [36]

	P/Tol	F/Be–$CHCl_3$ (1:1)	BL_1	
20β,21-Dihydroxypregn-4-en-3,11-dione	0.16	0.47	0.31	*
17α,20β,21-Trihydroxypregn-4-en-3-one	0.20	0.50	0.39	*
17α-Hydroxypregn-4-en-3,20-dione	3.11	1.85	1.27	I–KI : blue*
				$SbCl_3$
5α-Pregnan-3β,17α,20α-triol	0.30	1.06	1.11	reddish**
5β-Pregnan-3α,17α,20α-triol	0.40	1.07	1.08	blue**
5α-Pregnan-3β,17α,20β-triol	0.42	1.28	1.15	reddish**
3β,17α-Dihydroxy-5α-pregnan-20-one	1.12	1.68	2.13	**

* UV + ** UV —

TABLE 43

R_S-VALUES (S = CORTEXONE) OF C_{21}-STEROIDS AND THEIR ACETATES (UV +, BT +) [36, 295]

	P/Tol	F/Cy–Be (1:1)	B_3	A^{380}	
Corticosterone	0.24	0.04	0.07		
17-Hydroxycortexone	0.14	0.05			
11-Dehydrocorticosterone	0.55	0.13	0.15		
1-Dehydrocortexone	0.77	0.60	0.64	0.38	
Cortisone acetate	0.34	0.08	0.11		
Corticosterone acetate	0.84	0.51	0.61		
17-Hydroxycortexone acetate	0.79	0.67	0.68		
11-Dehydrocorticosterone acetate	1.11	0.96	0.81		
Cortexone acetate	1.33	1.66	1.62	2.48	
19-Hydroxycortexone-21-acetate	0.45	0.14	0.12		alkali-fluorescence
19-Hydroxycortexone-19-acetate	0.97	0.56	0.45		blue-green
19-Hydroxycortexone-19,21-diacetate	1.25	1.51	1.19	1.00	
6β-Hydroxycortexone-diacetate		1.57		1.45	
6α-Hydroxycortexone-diacetate			1.48	1.78	

References p. 186.

TABLE 44

R_S-VALUES (S = CORTEXONE) OF C_{21}-STEROIDS (UV —, BT + AND UV —, BT —) [36]

	P/Tol	F/Cy–Be (1:1)	B_3	
3α,17α,21-Trihydroxy-5β-pregnan-20-one	0.36	0.21	0.33	*
Allo-dihydro-corticosterone (5α)	0.49	tailing	0.24	*
3β,21-Dihydroxypregn-5-en-20-one	0.47	0.77	0.68	*
Allo-tetrahydrocortexone (3β,5α)	0.48	0.87	0.79	*
Dihydro-11-dehydrocorticosterone (5β)	0.75	0.32	0.35	*
Allo-dihydro-11-dehydrocorticosterone (5α)	0.80	0.33	0.37	*
21-Hydroxy-5β-pregnan-3,6,20-trione	0.81	0.12		alkali-fluorescence yellow! *
3α-Hydroxy-5α-pregnan-11,20-dione	0.88			1.10 (F/Be) **
3β-Hydroxy-5α-pregnan-20-one	1.05	1.70		**
3β-Hydroxypregn-5-en-20-one	1.04	1.87	1.37	2.96 ($A^{38°}$) **

* BT + ** BT —

TABLE 45

R_S-VALUES (S = CORTEXONE) OF C_{21}-STEROIDS AND DERIVATIVES (UV +, BT —) [36]

	P/Tol	F/Cy–Be (1:1)	B_3	$A^{38°}$	
17α-Hydroxyprogesterone	0.73	0.93	0.78	0.64	
11-Ketoprogesterone		1.35	1.09	1.23	
Progesterone	1.28	1.86	1.66	4.78	alkali-fluorescence weak
1-Dehydroprogesterone		1.84	1.41	2.50	alkali-fluorescence negative
20α-Hydroxypregn-4-en-3-one	0.88	1.50	1.17	1.16 *	
20β-Hydroxypregn-4-en-3-one	0.99	1.59	1.29	1.82 *	
3.11-Dioxoetiochol-4-enic acid methyl ester	1.21	1.78	1.38	2.08	
20 → 18 Lactone of 18-hydroxy-3-oxoetiochol-4-enic acid		0.90			
20 → 18 Lactone of 11β,18,18-trihydroxy-3-oxo-etiochol-4-enic-acid (aldosterone lactone)		0.47	0.36		
18 → 11 Lactone of 11β hydroxy-3,20-dioxopregn-4-en-18-oic acid		0.32	0.29		

* 22° C

BUSH [105, 177, 181] and SAVARD [180, 232], which have already been discussed individually in another part of the present chapter. The BUSH-technique has also been varied in that, in order to avoid the equilibration, the paper is wetted directly with a mixture of acetone and water (7:3) and water-saturated mixtures of hexane, benzene, toluene and alcohols are used as the mobile phase [345]. The original method has however been able to maintain its place to the present time. A number of variations of the BUSH-system, especially for strongly polar corticosteroids, have been given by VON PECHET [346]; in these, the paper is also first impregnated with the aqueous phase.

EBERLEIN AND BONGIOVANNI [347] have introduced some interesting variations

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(*cf.* Table 46). They equilibrate at a temperature about 10° higher than that at which the chromatography is performed. In our experience, however, more uniform results are obtained when the temperature is maintained constant for the whole time; it is advantageous, as in the case of BUSH-systems to work at approximately 38°.

TABLE 46

SOLVENT SYSTEMS OF EBERLEIN AND BONGIOVANNI [347]

NB_1	Isooctane–toluene–methanol–water	(25:25:35:15)
NB_2	Isooctane–toluene–methanol–water	(16.5:33.5:30:20)
E_1	Isooctane–methanol–water	(50:45:5)
E_2B	Isooctane–*t*-butanol–water	(50:25:45)
E_4	Isooctane–*t*-butanol–methanol–water	(50:22.5:22.5:5)
E_5	Isopropyl ether–*t*-butanol–water	(40:25:30)
E_9	Hexane–*t*-butanol–methanol–water	(50:22.5:22.5:20)

Of these new systems the one designated as E_2B proved particularly useful for corticosteroids (see Tables 37–39) since the sequence of R_S-values is in part different from that in other systems (*cf.* also [305]). Ethylene glycol/toluene instead of propylene glycol/toluene has recently been used again for special purposes [348], whereas water-free xylene–methanol systems [349] offer no visible advantage. For weakly polar $C_{21}O_2$- and $C_{21}O_3$-steroids AXELROD [231] has used formamide/decalin and butane-1,3-diol/methylcyclohexane, besides the systems mentioned above. The rate of migration of highly polar corticosteroids in several common systems is given in [350] in cm/h. For the relative migration rates of aldosterone and its acetates, *cf.* [295] and [351]. GRAY *et al.* [352] give, among others, the following R_F-values in BUSH-system B_5:

Prednisolone	0.22
Cortisol	0.31
Prednisone	0.44
Cortisone	0.46

Even more marked differences between Δ^4- and 1:4-dien-3-ketosteroids are obtainable with several ZAFFARONI-type systems. Other systems which have been used for corticosteroids are butanol–water [335] and heptanol–water [206, 207], the latter as a reversed phase system, but these systems are for one reason or another inferior to most others. ZANDER *et al.* [304] have investigated a series of BUSH-variations for the chromatography of relatively weakly polar steroids, particularly with reference to their suitability for progesterone (see Table 47). They have also worked out the optimal conditions for identifying progesterone, and on this basis developed a method for determining it. Metabolites of progesterone, particularly pregnandiols and pregnantriols, can for example, be separated in propylene glycol/toluene, BUSH B_4, or toluene/isooctane–methanol–water (15:5:16:4) and detected [242, 256, 353] with antimony trichloride, zinc chloride, or trichloroacetic acid [242]. Tables 48 and 49 give some R_F-values and color reactions.

For quantitative determinations, *cf.* Section 1 i (p. 163) and [354]. There

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TABLE 47

R_F-VALUES OF RELATIVELY WEAKLY POLAR STEROIDS AND THEIR ESTERS AS A FUNCTION OF VARIOUS CHROMATOGRAPHIC CONDITIONS [304]

System	*A (Bush)**	*a*	*b*	*c*	
Stationary phase	*80% methanol*	*80% methanol*	*70% methanol*	*80% methanol*	*80% methanol*
Mobile phase	*light petroleum 80–100°*	*ligroin 90–100°*	*n-hexane*	*light petroleum 55–65°*	*light petroleum 55–65°*
Technique	*descending*	*descending*	*descending*	*ascending*	*ascending*
Temperature	*34°*	*37°*	*37°*	*37°*	*37°*
Paper	*Whatman No. 4*	*Schleicher and Schüll 2045b*	*Schleicher and Schüll 2045b*	*Whatman No. 4*	*Whatman No. 1 Schleicher and Schüll 2043a, b, 2040b*
Equilibration	*up to 12 h*	*1–12 h*	*1–6 h*	*1 h*	*1 h*
Development	*45 cm in 100–180 min*	*20 cm in 120 min*	*20 cm in 120 min*	*19–25 cm in 60 min*	*19–25 cm in 60 min*
Testosterone	0.40		0.15	0.13	0.08
17β-Methylandrostendiol				0.16	0.11
17α-Methyltestosterone				0.28	0.18
Dehydroepiandrosterone		0.38	0.36	0.29	0.19
17β-Ethylandrostendiol				0.31	0.21
Isoandrosterone			0.36	0.33	0.29
Δ^4-Androstendione	0.70		0.39	0.34	0.25
17β-Estradiol monoacetate				0.36	0.25
Cortexone acetate	0.70	0.54	0.50	0.42	0.27
Δ^1-Androstendione				0.50	0.37
Androsterone		0.60	0.63	0.51	0.38
3β-Hydroxy-5α-pregnan 20-one				0.53	0.38
Δ^5-Pregnenolone		0.61	0.65	0.54	0.31
17β-Estradiol diacetate				0.61	0.49
Progesterone	0.85	0.72	0.73	0.68	0.51 **
$\Delta^{11,12}$-Dehydroprogesterone		0.72	0.73	0.68	0.51 **
17β-Estradiol monobenzoate				0.81	0.65
Δ^5-Androstendiol monoacetate				0.85	0.70
Dehydroepiandrosterone acetate		0.92	0.92	0.93	0.81
Δ^5-Androstendiol diacetate		0.93	0.93		
Δ^2 or Δ^3-Androsterone				1.00	0.88
Δ^5-Pregnenolone acetate				1.00	0.94
Cholesterol		0.93	0.94		

* This column presents the original values of BUSH in system A, for purposes of comparison.
** 0.38 with Schleicher and Schüll-paper 2045b.

exist numerous possibilities for evaluation on the basis of UV-absorption, reduction reactions, alkali-fluorescence etc., especially in cases where the spot can be located radiochemically [160, 289, 355]. The densitometry of formazan spots directly on the chromatogram, after it has been rendered transparent, is also worthy of mention [235]. It is possible only to refer to a number of further applications, whose study is nonetheless much to be recommended for the paper chromatographic techniques; these include paper chromatographic analysis of corticosteroids and their metabolites

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TABLE 48

AVERAGE R_F-VALUES AND COLOR REACTIONS OF PROGESTERONE-METABOLITES [353]

	P/Tol	B_4	UV-Fluorescence	
			$SbCl_3$	$ZnCl_2$
5β-Pregnan-3α,17α,20α-triol	0.08	0.44	red	blue
5α-Pregnan-3β,20α-diol	0.31	0.77	rose	blue
5β-Pregnan-3α,20α-diol	0.32	0.73	yellow orange	blue
5α-Pregnan-3α,20α-diol	0.35	0.71	rose	blue
5α-Pregnan-3β,20β-diol	0.41	0.78	rose	blue
17α-Hydroxyprogesterone	0.59	0.79	blue	blue
3β-Hydroxypregn-5-en-20-one	0.78	0.88	red	rose
3β-Hydroxy-5α-pregnan-20-one	0.86	0.89	blue	o

TABLE 49

R_F-VALUES OF PREGNANTRIOLS AND SIMILAR STEROIDS IN THE SYSTEM ISOOCTANE–TOLUENE–METHANOL–WATER (5:15:16:4) 22° C [242]

	R_F	Color reaction with trichloroacetic acid	
		in daylight	in UV-light
5β-Pregnan-3α,17α,20α-triol	0.33	blue	blue
5β-Pregnan-3α,17α,20β-triol	0.42	blue	blue
5α-Pregnan-3α,17α,20α-triol	0.38	blue	beige-gray
5α-Pregnan-3α,17α,20β-triol	0.48	blue	beige-gray
Pregn-5-en-3β,17α,20α-triol	0.26	violet	rose
Pregn-5-en-3β,17α,20β-triol	0.33	violet	rose

Corresponding compounds with 11-keto groups do not always give color reactions.

from urine [322, 333] and beef adrenals [280], determination of aldosterone in adrenals [139] or in urine [210, 305], and isolation of corticosteroids [295]. Finally, we may mention the following interesting examples of applications: identification of adrenal cortical hormones in human plasma [341], in human adrenals and adrenal tumors [323], isolation of aldosterone from urine [356], identification of corticosteroid-metabolites in urine [296], and chemical determination of progesterone [215].

(e) *Glycosides and aglycones (cardenolides, bufadienolides)*

Paper chromatography has yielded many results in this field, too; consequently, a large number of studies have appeared in which the chromatographic methods applicable to these highly polar substances have been continuously improved. Besides many aqueous systems, the ZAFFARONI- and BUSH-systems developed for use with steroids have also been applied. Chromatography on sheets has generally been preferred to that on strips.

(1) *Digitalis- and Strophanthus glycosides and aglycones.* A useful method was reported by SVENDSEN AND JENSEN [245] as early as 1950. They used chloroform–

methanol–water mixtures (I–III in Table 50) for chromatography, and trichloroacetic acid as a test reagent; the reaction is much more sensitive for purpurea glycoside B and digilanid B and their cleavage products (intense blue) than for the corresponding A-glycosides and cleavage products (reddish); digilanid C, digoxin and digoxigenin give steel-blue colors. These systems allow the application of only 5 μg of each substance, because of the tendency to tailing. The method has since been improved by the use of the ZAFFARONI-system formamide/benzene–chloroform (4:6) and of several color reactions like antimony trichloride, DNB, picric acid, sodium nitroprusside, etc. (*cf.* test reactions, Section 1 e, p. 151, [228]), and by fluorimetric determination *in vitro* of the eluates. Formamide/chloroform and benzene–chloroform mixtures were introduced as early as 1951 by SCHINDLER AND REICHSTEIN [229] for the chromatography of many glycosides and aglycones from Strophanthus seeds. The authors give relative migration velocities and note that many monoglycosides migrate faster than the aglycones. HEFTMAN *et al.* [234, 243] also use ZAFFARONI-systems like formamide/

TABLE 50

R_F-VALUES OF CARDIAC GLYCOSIDES AND AGLYCONES [228, 245, 248, 367]

	I	*II*	*III*	*IV*	*V*	*VI*	*VII*	*VIII*	*IX*
Digitalinum verum					0.95	*F*			0.16
Gitorin					0.78	*F*			
Digilanid C	0.08	0.12	0.15		0.86	*F*	0.25		0.33
Digilanid B	0.14	0.18	0.25		0.66	*F*	0.10		0.47
Digilanid A	0.37	0.46	0.49		0.28	*F*	0.03		0.85
Deacetyldigilanid C	0.01	0.01	0.02						0.13
Deacetyldigilanid B	0.02	0.03	0.05		0.73	*F*			0.19
Deacetyldigilanid A	0.07	0.10	0.15		0.39	*F*			0.50
Strospeside								0.75	0.61
Digoxin	0.68	0.75	0.76		0.29	0.72		0.16	
Digoxigenin	0.58	0.64	0.61					0.32	
Gitoxin	0.76	0.81	0.82	0.40	0.18	0.55		0.29	
Gitoxigenin	0.82	0.84	0.84	0.80	0.17	0.52		0.42	
Acetyldigoxin-α								0.39	
Acetyldigoxin-β								0.45	
Acetylgitoxin-α					0.08	0.41		0.59	
Acetylgitoxin-β								0.64	
Digitoxin	0.88	0.91	0.90	0.68	0.03	0.27		0.69	
Digitoxigenin	0.92	0.93	0.92	0.91	0.03			0.81	
Acetyldigitoxin-α and -β					0.02	0.21		0.90–0.95	
Oleandrin								0.93	
Oleandrigenin								0.72	
Nervifolin								0.55	
Cymarin								0.55	
Allocymarin								0.48	
Strophanthidin								0.40	
Allostrophanthidin								0.32	
Isostrophantidin								0.76	
Strophanthidol								0.26	
Cymarol								0.36	
Emicymarin								0.11	
Alloemicymarin								0.07	

See text for solvent systems I–IX. *F* = solvent front.

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benzene and propylene glycol, toluene for chromatograms where the solvent front is allowed to run off the paper. The distances of migration of several cardenolides obtained in this fashion are given in Table 51 and a graphical representation in [234]; color reactions with trichloroacetic acid and Tollens-reagent are given in [243]. HASSALL AND MARTIN [326] reported in 1951 on their experiments with various mixtures of water, esters, ketones, alcohols, chloroform and sodium benzoate, some of which constitute useful systems (*cf.* also JAMINET [241, 246]). There followed further variations of these systems (acetyl derivatives of gitoxigenin [358], digitalis

TABLE 51

DISTANCES OF MIGRATION (in cm) OF CARDIAC GLYCOSIDES, AGLYCONES AND THEIR ACETATES [243]

Substance	*F/Benzene*		*P/Toluene*	
	24 h	*48 h*	*24 h*	*48 h*
Periplogenin	2.8	4.2	0.7	2.7
Periplogenin acetate	21.6	43.6	6.9	16.9
Alloperiplogenin acetate	15.0	32.0	6.6	
Alloperiplocymarin	11.8	28.9	2.5	7.1
Periplocymarin	16.2	38.9	3.8	10.0
Emicymarin	0.8	1.1	0.7	3.0
Emicymarin acetate			13.6	23.4
Alloemicymarin	1.0	1.4	1.0	2.5
Cymarol	2.5	3.6	1.0	3.0
Strophanthidol	2.0	2.6	0.6	1.4
Strophanthidol acetate	8.3	15.8	4.7	12.0
Strophanthidin acetate	4.1	6.1	2.8	7.1
Cymarin	4.0	6.2	2.0	5.1
Cymarin acetate			15.1	
Sarmentocymarin	3.3	4.1	0.8	3.1
Sarmentogenin	2.5	2.5	1.0	2.0
Sarmentogenin acetate			15.5	25.3
Sarmentoside A	2.3	2.3	0.7	1.3

TABLE 52

R_F-VALUES AND COLOR REACTIONS OF SOME CARDIAC GLYCOSIDES IN THE SYSTEM METHANOL–CHLOROFORM–WATER (10:10:6) [273]

Substance	R_F	*Anisaldehyde–glacial acetic acid–sulfuric acid reagent*		*Raymond reagent*	*Legal reagent*
		visual light	*UV-light*	*visual light*	*visual light*
Diginin	0.24	green blue	dark blue	blue	red
Digitoxin	0.51	green blue	dark blue	blue	red
Gitoxin	0.70	green blue	light blue	blue	red
Saponin	0.77	yellow	dark violet	—	—
Gitoxigenen	0.84	—	light blue	blue	red
Purpurea glycoside A	0.84	green blue	light blue*	blue	red
Purpurea glycoside B	0.93	green blue	light blue	blue	red

* The dark blue fluorescence of purpurea glycoside A is masked by the light blue fluorescence of gitoxigenin, which has the same R_F-value.

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[244, 359, 360] in some of which silicone-impregnated hydrophobic paper was used [203, 273] with aromatic aldehydes as reagents [203, 273, 276], *cf.* Table 52.

BUSH's system B had meanwhile been successfully applied to these separations [208], see Table 53. Water saturated with butanol has also on occasion yielded good chromatograms [257]. The R_F-values of non-glucose-containing substances of the Purpurea- and Lanata-group are very small to be sure; one therefore either allows the solvent front to run off the paper, or one adds glacial acetic acid or methanol, *i.e.*, butanol–glacial acetic acid (or methanol)–water (5:1:5); the aqueous phase is suitable for the Purpurea- and Lanata-group, and the butanol phase for the Strophantus-group. A quantitative analysis appears to be possible, too.

TABLE 53

R_F-VALUES OF SOME AGLYCONES AND DERIVATIVES IN THE BUSH-SYSTEM B_5 [208]

Substance	R_F
"β"-Anhydrodigitoxigenin	0.91
"α"-Anhydrodigitoxigenin	0.63
"β"-Anhydrodigoxigenin	0.73
"β"-Anhydrodigoxigenone	0.59
"β"-Anhydrosarmentogenin	0.69
"β"-Anhydrouzarigenin	0.94
Digitoxigenin	0.76
Digitoxigenin acetate	0.89
Digoxigenin	0.075
Digoxigenin diacetate	0.70
Digoxigenone	0.68
Gitoxigenin	0.26
Gitoxigenin diacetate	0.88
Periplogenin	0.43
Sarmentogenin	0.10
Sarmentogenin diacetate	0.85
Sarnovide mono(?) acetate	0.72
Sarverogenin	0.40
Strophanthidin	0.16
Strophanthidol	0.05
Uzarigenin	0.83

For the determination of the components of samples of digitalis SILBERMAN AND THORP [287] used ethyl acetate–benzene–water with 1.5–7.5% of alcohol, followed by fluorescence photography and densitomitry. The system was later much improved by the additional use of chloroform [361]. Such systems were then altered in many ways, and used, according to need, for group separations or for the isolation of individual digitalis glycosides and genins [363]. More complicated solvent mixtures [364] are hardly satisfactory.

TSCHESCHE *et al.* [248] obtained good results with a reversed phase system. In this case the paper is impregnated with the organic phase of the mixture octanol–pentanol–water–formamide 5:6:8:2 (V) or 6:2:1:4 (VI) or, *e.g.* pentanol–water 1:1 (VII) and the aqueous phase is used as the mobile one (see Table 50). The quantitative evaluation is made on the eluates with xanthydrol. For cardenolides from *Convallaria*

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majalis, the above systems were used, and also isobutanol–water (VIII) and butanol–pyridine–water 3:1:3 (IX, Table 50) according to the following schema [362]:

Substances	*Systems*				
	V	*VI*	*VII*	*VIII*	*IX*
Genins + anhydrogenins	+	+			
Acetates	+				
Vallarotoxin	+				
Majaloside, convallatoxin, convallatoxol			+	+	
Convalloside, convallatoxoside, glucosido-convalloside			+	+	+

Developing their methods still further, REICHSTEIN *et al.* [365] discovered some excellent systems for strongly polar glycosides and aglycones. Paper wet with water served as the stationary phase and *n*-butanol or *n*-butanol–toluene mixtures as the mobile one. The latter system is especially suitable for convallatoxin, digitalinum verum monoacetate, gofruside, frugoside, nigrescigenin, odorobioside-G monoacetate, odorotrioside-G monoacetate, sargenoside diacetate, thevebioside, and thevetin, and the water–butanol system for acolongifloroside K, ouabain, sarmentoside A, *k*-strophanthin-β, *k*-strophanthoside, and uzarin. Slight tailing sometimes occurs. The results have been reproduced graphically.

These systems have since been employed by the REICHSTEIN-group with great success for the detection of many other glycosides, as may be seen from recent volumes of *Helvetica Chimica Acta*. As particularly instructive examples we mention here only the isolations from *Strophanthus ledienii* [87, 89], *Erysinum crepidifolium* [90] and *Glossostelma spathulatum* [366]; a large number of color reactions are also described in these papers.

For the complete separation of digitalis glycosides and aglycones by ascending chromatography, KAISER [367] lists two new solvent mixtures of the ZAFFARONI-type: formamide/xylene–methylethyl ketone (1:1) for glycosides of the complexity of digitoxin and for aglycones, and formamide/chloroform–tetrahydrofuran–formamide (5:5:0.65) for the primary glycosides (see VIII and IX in Table 50). System VIII is, moreover, suitable for many other cardiac glycosides which are sparingly soluble in water. Other similar systems have been described for specific separations and for possible identification of the sugars occurring in cardiac glycosides. The α- and β-forms of acetyldigitoxin, acetylgitoxin and acetyldigoxin can be separated by repeated chromatography in formamide/xylene–methylethyl ketone (2:1 or 3:1). For a variant with ethylene glycol/chloroform, *cf.* [368].

(2) *Glycosides and aglycones of Scilla-Bufo-type.* Solvents like those described above under cardiac glycosides are suitable for the chromatography itself. The following variations of the ZAFFARONI-type have proved especially advantageous: propylene glycol–water (4:1)/benzene–petroleum ether (1:1) or benzene–chloroform (1:1), and also formamide/benzene–chloroform (6:4) [96, 369]; the results have been reported in part graphically [96], and in part by giving the R_F-values [369]. The

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identification is made primarily by the color reaction with antimony trichloride (the color tests are reported in [96, 369] or by photocopy with a filter at 300–350 mμ, which specifically detects the cumalin (doubly-unsaturated lactone) ring which is characteristic of the Scilla-Bufo type [225]. The most recent compilation is given in [385].

(f) *Saponins and sapogenins*

Aqueous systems have proved best for saponins. Some of these are acid, *e.g.* *n*-butanol–acetic acid–water (4:1:5) [275, 286], some neutral, like chloroform or benzene mixed with various aqueous alcohols (butanol, isoamyl alcohol) and some even basic, like benzene–butanol–pyridine–water (10:4:1:5) [275]; antimony trichloride, aromatic aldehydes or periodate-permanganate (*cf.* test reactions 1 e, p. 151) serve as test reagents.

The reports on sapogenins are somewhat more numerous. VON SANNIÉ *et al.* [259, 260, 370] first proposed benzene–chloroform–glacial acetic acid systems, which are probably chiefly useful for separation into various groups. Neutral aqueous systems like petroleum ether–toluene–ethanol–water in ratios of 0–40:40–0:1–3:9–7 [247] appear to give better results, however, partly also for sapogenin acetates. Finally, such ZAFFARONI-type systems as carbitol or formamide as the stationary phase and hexane–octane or benzene as mobile phase [274] have also proved entirely suitable, the solvent front being sometimes allowed to flow off the paper, while a dye is used for a marker. In many cases, the use of both types of systems is advantageous in this field (see also [383]). The sapogenins can be detected *e.g.* with antinomy trichloride, trichloroacetic acid, aromatic aldehydes, or biologically (even on the paper) by their ability to hemolyse blood [247].

(g) *Bile acids, steroid carboxylic acids*

Whereas the neutral systems of BUSH or ZAFFARONI, which are usually used in steroid chromatography, are suitable for esters of the carboxylic acids (depending upon their polarity) (*cf.* also [371]), acid or basic systems should be used for the free acids. Examples of basic systems are *n*-propanol–NH_4OH–water (90:2:8) or better *n*-propanol–ethanolamine–water (90:5:5) [372], collidine–water (10:3.5) in an atmosphere of ammonia [261] or isoamyl alcohol saturated with 3 *M* ammonium carbonate or 2% ammonia [271]. The following acid systems have proved useful, for example: stationary phase 70% acetic acid (impregnated in the paper), mobile phase isopropyl ether–heptane 6:4, 2:8, or 8.5:1.5 [251, 252]; butanol saturated with 3% aqueous acetic acid [251], 80% acetic acid [271] and toluene–acetic acid–water 1:1:2 [240]. Most of these systems are also useful for the conjugated taurocholic and glycocholic acids; ascending or descending chromatography can be used. Test reagents may be, among others, antimony trichloride, phosphoric acid, phosphomolybdic acid, aromatic aldehydes, and in special cases, iodine in potassium iodide (*cf.* test reactions, Section 1 e, p. 151, and especially the data on numerous acids and esters in [271].

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Bile acids with reactive keto groups can be chromatographed as Girard-derivatives, [373]; it is usually preferable, however, to work with the free acids or their esters. For the quantitative determination of bile acids separated on paper, *cf.* [374, 375]; for radioautographic determinations, [261].

(*h*) *Conjugates of steroids*

As in the case of the bile acids, these compounds are also acid (conjugation with glucuronic- or sulfuric acid) and several aqueous systems appear quite suitable. Particularly noteworthy are *n*-butyl acetate–*n*-butanol–10% acetic acid (or 10% formic acid) in the ratio 4:1:5, or *n*-butyl acetate–methanol–0.1 *M* barbiturate buffer, pH 8.2 (3:1:1, monophasic) or ethyl acetate–*n*-butanol–0.2% aq. ammonia (7:1:8) or isoamyl alcohol–conc. NH_4OH–water (55:27:18). The R_F-values of a large number of glucuronides [250, 386] and sulfates [376, 386] of C_{19}-steroids, and progesterone- and corticosteroid metabolites are given in the literature. Water-saturated butanol or ethyl acetate–*n*-butanol–0.2 *N* acetic acid (9:1:10) are suitable for these compounds, including estrogen sulfates [263, 386]. There are many possible methods of identification, [386], including UV-absorption, *m*-dinitrobenzene, phosphomolybdic acid, antimony trichloride, etc., and in the case of sulfates, rhodizonic acid [376].

(*i*) *Aminosteroids and steroid alkaloids*

Steroids with basic substituents and alkaloids with steroid-like skeletons constitute the last class which we shall mention. For steroid amines which contain only one nitrogen atom butyl acetate saturated with water, and the reversed phase system with kerosine (paraffin oil) and aqueous alcohol have been proposed [194]. These systems also enable one to separate some 3α- and 3β-isomers. For the chromatography of alkaloids from *Holarrhenia antidysenterica* (conessine, solanine, etc.), these authors give the R_F-values in amyl alcohol–acetic acid–water (4:1:5; upper phase) or in paraffin oil/ethanol–NH_4OH–water (75:2:23; reversed phase system). These systems can be varied within wide limits; the system butanol–acetic acid–water (4:1:5; upper phase), among others, has been found very useful for the alkaloids mentioned [278]. The isomeric solanine and chaconine can be well distinguished [253] with ethyl acetate–acetic acid–water (3:1:3), 15% of alcohol by volume being added to the upper phase. For the hydration products of veratrin alkamines, paper impregnated with *M* KCl has been used, with butanol saturated with 2% HCl as the mobile phase [279]; for the hypotensive alkaloids from *Veratrum album* (protoveratrine, etc.) another acid system has been used: ethylene chloride–acetic acid–water (49.5:1:49.5) or a different solvent instead of the ethylene chloride [377]. Test reagents may be in part those used for steroids and in part those used for alkaloids; antimony trichloride, antimony pentachloride, phosphomolybdic acid, fluorescence reactions (the substances sometimes show a fluorescence of their own in UV-light) or potassium iodoplatinate or iodobismuthate are often employed (*cf.* test reactions, Section 1 e, p. 151).

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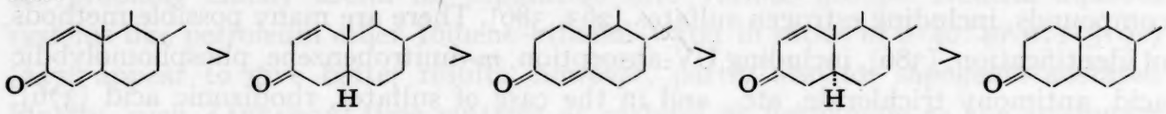
3. Chemical structure and chromatographic behavior

A number of rules for the behavior of steroids in partition chromatography can be derived (and partially confirmed) from systematic investigations [178, 181, 232] and Tables 30–49. In a given system the R_F-value depends primarily upon the number of C- and O-atoms, the ratio of which determines the polarity of the substance, up to a certain point. If the oxygen-containing groups are equivalent, the polarity decreases (and the R_F increases) in the order $C_{18}O_2 > C_{19}O_2 > C_{21}O_2$. Likewise, propionates migrate faster than the corresponding acetates.

Besides the C/O ratio the R_F depends chiefly upon the nature of the oxygen-containing groups; the polarities are as follows

$$>\!COH > >\!C=O > -COOR > >\!C-O-C\!<$$

Lactones generally have a greater effect than do ordinary esters. The effect of the ketone group varies according to its position in the molecule; the polarity of ketones runs C_3 or $C_{20} > C_{11} > C_6$. It is also definitely affected by conjugated double bonds, *viz.*:



Although the manner of joining the rings (A/B *cis* or *trans*) makes a difference in the Δ^1-3-ketones, this is hardly true any longer in the case of the saturated 3-keto compounds, where the 5α- and 5β-series have the same R_F-values. Isolated double bonds, *e.g.* $\Delta^{9(11)}$, do not have any significant influence upon the R_F-values either. On the other hand, Δ^5-3β-hydroxysteroids are usually somewhat more polar than the saturated compounds if there are not too many other oxygen atoms present in the molecule.

The effect of hydroxyl groups is particularly large and variable. It depends upon

1. the conformation (axial or equatorial)
2. steric relationships
3. mutual interactions with the chromatographic milieu.

It has been shown that all equatorial hydroxyl groups are more polar than axial ones (*cf.* Formulas II and III, p. 100) *e.g.*:

$$11\alpha > 11\beta; \qquad 6\alpha > 6\beta; \qquad 7\beta > 7\alpha; \qquad 17\beta > 17\alpha$$

Table 40 (p. 172) shows that the following relationships hold for the hydroxycortexones, for example:

for P/Tol: $16\alpha > 15\alpha > 19 > 11\alpha > 7? > 12\alpha > 18 = 6\beta > 14\alpha > 17\alpha > 8$ or $9 > 11\beta$

for F/Be-$CHCl_3$: $16\alpha > 15\alpha > 19 > 7? > 11\alpha > 18 = 6\beta > 12\alpha > 8$ or $9 > 14\alpha > 17\alpha > 11\beta$

From the literature, we have, for the monohydroxyprogesterones:

for P/Ligroin: $6\alpha > 16\alpha > 11\alpha > 6\beta > 14\alpha > 11\beta > 17\alpha > 21$.

These examples demonstrate that all equatorial hydroxyl groups (e) are more polar than axial ones (a), no matter whether they be primary or secondary. The differences within each group (e or a) are determined by steric factors among other

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things, and displacements within the group, caused by changing the solvent system, are explicable at least in part on the basis of the mutual interactions between the substances and the milieu. The equilibria of formation of hydrogen bonds between the substances, the mobile and the stationary phases, and the cellulose probably play a major role in this connection (*cf.* also [209] and the singular behavior in the system E_2B [347]). It is just such differences as these which are of the greatest importance for paper chromatographic identifications. Examples of changes in the order of substances on the chromatogram on changing the BUSH- or ZAFFARONI-systems can easily be found in the tables. Here we note only the classical case of aldosterone, which, especially capable as it is of forming intermolecular and intramolecular hydrogen bonds, shows, in contrast to cortisone or cortisol, quite varied R_F-values in different systems.

The steric relationship of rings A and B merits closer attention. The assertion which is frequently made, that steroids with the 5α-configuration (A/B *trans*) are less polar than the 5β-steroids, is certainly not true as stated. The polarity is also quite dependent upon the presence of axial or equatorial substituents in ring A. Whereas, as has been mentioned above, 5α- and 5β-steroids are practically inseparable when ring A contains no substituents, or only a 3-keto group, the situation is fundamentally altered when a hydroxyl group is present at C_3. Using the designations α and β for the hydroxyl group at C_3 and the H-atom at C_5, the following polarity sequence is definitely established by the tables (for C_{19}- and C_{21}-steroids).

$$3\beta,5\alpha \geqq 3\alpha,5\beta > 3\beta,5\beta \geqq 3\alpha,5\alpha$$

3-hydroxyl	equatorial	axial
	I	II

This sequence depends directly upon the conformation of the hydroxyl group and not on the ring function. The difference in polarity between Group I and Group II is usually considerable and sufficient for separability. The difference within each group is, on the average, less and depends fairly strongly upon other factors, such as the solvent system and the other substituents; it is not always sufficient for separation. The 20α-hydroxyl group in C_{21}-steroids is usually more polar than the 20β-group; exceptions may occur, depending upon other substituents and the solvent systems. As examples of such interactions we may mention that the contribution of 11β-hydroxyl groups to the polarity in ZAFFARONI-systems is greater than that of 20-hydroxyl groups, while the opposite is true in BUSH-systems [350]. The same rules hold for cardenolides [243] and for sapogenins [247, 259] as have been discussed above for the steroids. It is remarkable, however, that some monoglycosides run faster than their aglycones (*cf.* Table 50 [229]).

When one considers the relationships which have been found between constitution and behavior of steroids in partition chromatography, one should also consider the superimposed adsorption phenomena, which may be more or less marked, and which are difficult to predict in advance. Displacement effects observed with mixtures are also to be considered. While it is possible on the one hand that excellent possibilities for separation may exist because of the very great differences in polarity of different

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oxygen-containing groups, it is also possible on the other hand that steroids with very different oxygen contents may have the same R_F-values in certain systems. Triketones may behave like hydroxyketones, or triols like dihydroxyketones, for example; differences in C-content may also have a levelling effect. All the more valuable, therefore, is the possibility, referred to above on several occasions, of differentiating by using different solvent systems, various color tests, and additional physical-chemical properties (see Section 1 f, p. 159).

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PAPER CHROMATOGRAPHY OF CHLOROPLAST PIGMENTS

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The classical method of chromatography of chlorophylls and carotenoids on an adsorbent column is somewhat time-consuming and is more suitable for separating larger quantities of pigments. Therefore many authors have tried to develop a simpler method of separating these substances on paper. With the methods that have been described in the literature it is possible to analyse quantities of pigments of the order of 10^{-6} g. In recent years various modifications of these methods have been described and they have been used on a larger scale than before (more than 50 articles since 1952). Some of these articles have been reviewed in three communications[15,37,55]. In this paper we are attempting to give a more complete list of articles on paper chromatography of plastid pigments. Modifications of the methods that have been described in the literature are summarized in Tables 1–5 (pp. 196–205).

1. OLDER PUBLICATIONS

The first time a separation of plastid pigments on paper was mentioned, was in 1906. At that time the founder of chromatography, M. TSWETT[60], described a separation of pigments from an aqueous alcohol solution by capillary analysis. As a result he obtained five zones most of which were close together. At the bottom of the paper strip a zone containing a mixture of all the pigments was found, then came the following zones, in ascending order: carotenes, chlorophylls, xanthophylls. Capillary analysis, from a mixture of ethyl ether and 96% ethanol (1:1 to 1:2), was also used by KYLIN[34] for the detection of carotene, xanthophyll, phyllorhodin, fucoxanthin and chlorophyll in saponified extracts of higher plants and brown algae.

BROWN[10] experimented with the circular chromatographic method on blotting paper. The paper, 15 × 15 cm (untreated or impregnated with aluminium hydroxide), was sandwiched between two glass plates. The solution of pigments in carbon disulfide was placed on the paper through a 6 mm hole at the centre of the upper plate. The chromatogram was developed by adding the pure solvent drop by drop.

STRAIN (ref. 58, p. 77) briefly mentioned the separation of pigments on paper by developing with aqueous ethanol. The possibility of separating pigments on wide sheets of paper or on cylinders of paper was proposed by ARONOFF (ref. 3, p. 55; see [36]), the chromatograms being developed repeatedly with petroleum ether.

2. EVOLUTION OF THE METHOD SINCE 1952

Although MÁRKUS and ASAMI reported their experiments with paper chromatography of plastid pigments at two scientific meetings as early as 1951, and although ASAMI

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published a short article in Japanese in the same year[4], the method did not begin to develop favourably until 1952. At that time five independent groups of scientists published their papers dealing with this method. These communications came from BAUER[8] in Germany, MÁRKUS[38] in Hungary, ASAMI[5] in Japan, and from two French teams specially working on the separations of carotenoids, GRANGAUD AND GARCIA[23,24] and SPITERI AND NUNEZ[56]. The methods of these authors have been modified by many further workers. One- and two-dimensional techniques in ascending, descending and circular arrangements have been described. Different commercial brands and different types of paper have been used, sometimes after a preliminary treatment, such as drying, washing or impregnation. The chromatograms have been developed with polar and nonpolar organic solvents or their mixtures. In some experiments the atmosphere in the chromatograph chamber was specially prepared, *e.g.* by saturation with water vapour or vapours of petroleum ether; in some cases the chamber was filled with an inert gas (*e.g.* nitrogen). Chromatograms have been developed at laboratory temperature or at a somewhat lower temperature. A survey of the methods that have been described in the literature is presented in Tables 1 to 5. The developing solvents and mixtures examined, the types of paper used, the techniques of placing the extract on paper, the conditions of development (atmosphere, temperature, time of development) and plant material used are summed up in these tables. Satisfactory developing solvents are printed in bold face; the relative quantity of each solvent in the mixtures is given in parts by volume.

Communications describing the use of the method of BAUER[8], with or without slight modifications, are summarized in Table 1. Good results have been obtained especially on developing with the mixture petrol–petroleum ether–acetone (10:2.5:2), in which petrol is sometimes replaced by benzene. The methods of Japanese authors (Table 2), who used the Japanese paper Toyo No. 50 and developing mixtures containing toluene, occupy a somewhat exceptional position, since the quality of paper can influence the partition in a rather significant manner. Methods in which other developers than those derived from BAUER'S[8] mixture and other papers than Toyo paper were used, are summed up in Table 3. The remarkable article by MÁRKUS[38], in which the influence of various developers and various types of paper on the separation of pigments has been carefully examined, has rarely been cited. A specially arranged evacuated chamber and development with a five-component solvent mixture have been described by HAGER[27,28].

In Tables 1–3 a survey of methods based on the main principles of adsorption is presented. In Table 4 a summary is given of methods, which, according to their originators, are based on the principles of partition chromatography[15,16,48]. DOUIN[15,16] developed his chromatograms on paper moistened with water. He placed a perforated glass cylinder in the development chamber to ensure contact of the solvent (100% methanol) with water vapour. SERCHI, MICHI AND RAPI[48] used the circular development technique. Methods based on the principle of the so-called "reversed-phase chromatography" may also be found in Table 4. In this technique paper is impregnated with a nonpolar substance (triglycerides, vaseline, etc.), a strongly polar solvent

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being used as developer. The sequence of spots is approximately the reverse of that obtained with the usual type of paper chromatography, where at least small amounts of water (polar component) are held on the paper. The "reversed-phase" technique has been used by SPITERI AND NUNEZ[56] and STRAIN[59], and NUNEZ[40] (see Table 5) has resolved carotenoids by this method. STRAIN[59] has tested different techniques of preliminary preparation of cellulose paper and glass paper, as well as different types of separation (a–d in Table 4).

Data of the separation of only carotenoids from extracts have been collected in Table 5.

3. RESULTS OF THE CHROMATOGRAPHIC SEPARATION

Except in the case of "reversed phase" chromatography, the pigments are resolved with different solvents on one-dimensional chromatograms in the following general order (beginning from the starting point): chlorophyll b–chlorophyll a–pheophytins–carotenes. The greatest difficulties are caused by xanthophylls, which sometimes form one spot, while in other developing solvents several spots may arise (lutein–violaxanthin–neoxanthin and others). These spots lie in front of, between, or behind the spots of chlorophylls, often overlapping them, and thus interfering with the isolation of microquantities of pigments. The "R_F" values* of carotenoids depend on the number of oxygen atoms in their molecules: carotenoids containing fewer atoms of oxygen (less polar compounds) possess a greater "R_F" value[54]. Carotenes (without oxygen) run immediately behind the solvent front, followed by xanthophylls with two oxygen atoms in their molecules (*e.g.* lutein, zeaxanthin); the spot(s) of xanthophyll-epoxide(s) with four oxygen atoms (violaxanthin, neoxanthin, etc.) has (have) the smallest "R_F". Up to the present no successful developer has been found for resolving the carotene spot into α- and β-carotene. The pheophytins form one or two spots (pheophytin b and pheophytin a). Some authors[27, 28, 53, 54] have succeeded in separating pheophorbides as one or two spots. Chlorophyllides and similar hydrophilic compounds do not run with the solvent but remain at the starting point[5, 8, 27, 28, 49, 53, 54]. FOUASSIN[19, 20] separated Zn- and Cu-derivatives of chlorophyll and found that chlorophylls in which Mg was replaced by Zn moved quite similarly to normal chlorophylls a and b, whereas chlorophylls with Cu instead of Mg had "R_F" values similar to pheophytins. Double spots of chlorophylls have been observed on some chromatograms[5, 49, 57]. They have usually been regarded as isomers—chlorophylls a′ and b′, originating during the preparation of the extract or during the process of paper chromatographic separation. A colourless spot fluorescing in U.V. light has also been observed[36, 49].

For better resolution of some pigments, two-dimensional chromatography has sometimes been used[8, 36, 39, 45]. However, this technique is slower than one-dimensional chromatography, and is therefore not favourable for the unstable plant pigments.

Using "reversed-phase" chromatography the sequence of pigment spots is

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* "R_F" in this paper indicates only the approximate position of the spots on the paper, no definite value as is the case in the chromatography of amino acids and other compounds. Therefore quotation marks are used.

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TABLE

MODIFICATIONS OF THE

1 *Author*	2 *No. of dimensions*	3 *Ascend. (A) or descend. (D)*	4 *Paper, sort and dimension*	5 *Preliminary treatmen of the paper*
BAUER[8], 1952	1	(A or D)	Schl. & Schüll 2043 b (strips 10–15 cm long)	dried at 105°
	2		Schl. & Schüll 2043 b (10 × 10 cm)	
SIRONVAL[52,53,54], 1953, 1954, 1957	1	D	Whatman No. 1 (45 × 5 cm)	
BLAAUW-JANSEN[9], 1954	1	A	Whatman No. 4	buffered at pH 6
ANDERSEN AND GUNDERSEN[1], 1955 GUNDERSEN AND FRIES[26], 1956	1	D	Whatman No. 1 (strip 50–70 cm long)	
RÖBBELEN[43], 1956	1	A	Schl. & Schüll 2230	
GAGE AND ARONOFF[22], 1956	1	D	Schl. & Schüll 539	
MÜLLER[39], 1957	2	A	Schl. & Schüll 2043 bM (18 × 18 cm)	

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1*

METHOD OF BAUER[8] (1952)

6 *Placing the sample on paper; solvent used*	7 *Developing solvent or solvent mixture***	8 *Conditions of development****	9 *Plants analysed*
	monochlorobenzene **toluene** **petrol–PE–Ac (10:2.5:2)** **petrol–PE–Ac–Me (10:2.5:1:0.25)** Ac petrol PE Me 1st dim.: **petrol–PE–Ac (10:2.5:2)** 2nd dim.: **petrol–PE–Ac–Me (10:2.5:1:0.25)** (in mixtures PE can be replaced by monochlorobenzene or by toluene)		*Tradescantia albiflora*
a piece of plant tissue is squeezed on the starting point	**benzene–PE–Ac (10:2.5:2)**	S.F.–S.P.: 40 cm atmosphere of PE +23°	*Heracleum* *Pelargonia* *Fragaria*
acetone extract			
ethyl ether extract	**benzene–PE–Ac (10:2.5:2)**		*Chlorella vulgaris*
ethyl ether or acetone extract	**benzene–PE–Ac (10:2.5:2)**	15° atmosphere of PE	pigments in gyttja pith and xylem of: *Corylus avellana* *Fagus silvatica* *Salix caprea*
acetone	**benzene–PE–Ac (10:2.5:2)**		*Arabidopsis thaliana*
	benzene–PE–Ac (10:2.5:2)	atmosphere of PE	*Soja hispida*
acetone	1st dim.: **petrol–PE–Ac (10: 2.5:2)** 2nd dim.: **petrol–PE–Ac–Me (10:2.5:1:0.25)**		*Abies alba*

* Abbreviations used in Tables 1 to 5: PE = petroleum ether; Me = methanol; Ac = acetone.

** Satisfactory mixtures are printed in bold face; other mixtures that have been tested are also given.

*** Duration of the development; distance of solvent front from the starting point (S.F.–S.P.); temperature; atmosphere in the chamber.

TABLE
METHODS OF

1	2	3	4	5
ASAMI[5,7], 1952, 1955	1	A	Toyo filter paper 50 (1.2 × 35 cm)	
CHIBA AND NOGUCHI[14], 1954 CHIBA[13], 1955	1	A	Toyo filter paper 50	
KATAYAMA AND SHIDA[32,33], 1956	1	A	Toyo filter paper 50	

TABLE
OTHER

1	2	3	4	5
MÁRKUS[38], 1952	1	A or D	**Schl. & Schüll 602 e.h.** (the following papers were also tested: Hungarian paper, no brand mentioned Schl. & Sch.: 595, 597, 598 Swedish paper, no brand mentioned Munktell OB Macherey-Nagel 640 D) (paper strips 20–25 cm long)	paper equilibrated with atmosphere of acetone
LIND, LANE AND GLEASON[36], 1953	2	A	Whatman No. 1 (23 × 23 cm)	washed with PE and dried
HARDER AND KOCH[30], 1954	1	A	Schl. & Schüll 2043 b (strip 3 cm wide)	dried at 60°

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2*

JAPANESE AUTHORS

6	7	8	9
Me–Ac (3:1)	**toluene** **carbon tetrachloride** **xylene** } **anhydrous or saturated with water** *n*-butanol isobutanol benzene chloroform } anhydrous or saturated with water Me 100 % or 80 % ethanol 100 % or 80 % phenol 80 % cyclohexanol Ac lutidine collidine petrol (b.p. 45–60°) ethanol–*n*-butanol (1:1) Me–ethanol (1:1) benzene–petrol (1:1)		*Trifolium repens*
ethyl ether	**toluene** **toluene–90% ethanol (200:1)**	time: 15 to 30 min	*Trifolium repens*
Me–Ac (3:1)	**toluene–PE (2:1)**	time: 40 min + 1 to + 2°	*Oryza sativa*

3*

METHODS

6	7	8	9
acetone	**petrol–Ac–Me (30:1:0.03)** **PE–Ac (20:1)** PE (b.p. 35–40°) PE–Me (20:1) Pe–Ac–toluene (20:1:1) PE–Me–benzene (60:3:1) petrol (b.p. 60–70°) petrol–Ac (20:1) petrol–chloroform (20:1) petrol–ethyl ether (20:1) petrol–Ac–benzene (40:2:1) petrol–Me–benzene (40:1:1) petrol–Ac–*n*-butanol (20:1:0.3)	time: 2–3 h S.F.–S.P.: 20–25 cm 20° atmosphere of PE	*Solanum lycopersicum* *Spinacia oleracea*
PE	1st dim.: first **acetone** then **PE** finally **PE–propanol (99:1)** 2nd dim.: **PE–chloroform (3:1)**	S.F.–S.P.: 1 cm 20 cm 20 cm 16 cm time: 1 + 1 h	*Soja hispida*
PE (xanthophylls have previously been eluted with methanol)	First **toluene** then **PE–isobutanol (100:15)**	S.F.–S.P.: 8 cm time: 15 + 3 min	*Pedinomonas tuberculata* (Chlorophyceae)

* For abbreviations and column headings see Table 1.

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TABLE 3

1	2	3	4	5
BUKATSCH[11], 1954	1			
FOUASSIN[19,20], 1954	1	D	Whatman No. 1 (40 cm long)	
SPORER, FREED AND SANCIER[57], 1954 FREED, SANCIER AND SPORER[21], 1954	1	A	Whatman No. 1 (strip 3 cm wide)	impregnated with a solution of sucrose (0.18 g/ml in distilled water) and dried at 100°
SAPOZHNIKOV, BRONSHTEIN AND KRASOVSKAYA[45], 1955	1	A	Chromatographic paper No. 1, manufactured in U.R.S.S. or usual filter paper (cylinder from roll 16 × 16 cm)	washed with PE
	2			
LEFORT AND SIGNOL[35], 1955	1	A	Whatman No. 1 (2 × 26 cm)	dried at 105°
HAGER[27,28], 1955, 1957	1	A	Schl. & Schüll 2071 (cylinder)	dried at 50°
SHLYK[50], 1956	1	A	normal filter paper or paper No. 109-71 manufactured by Leningrad papermill No. 2	
JIRÁČEK[31], 1957	1	A	Whatman No. 1 (25 × 46 cm or 6 × 46 cm)	
ŠESTÁK[49], 1958	1	D (A also)	Whatman No. 1	no special treatment, or impregnated with sucrose (0.18 g/ml) and dried

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LE 3 *(Continued)*

6	7	8	9
acetone	**PE** **benzene**		*Capsicum*
PE	**PE–benzene–Ac (10:1.5:1)**	time: 3–4 h 21° S.F.–S.P.: 35 cm	*Spinacia oleracea* *Rumex* sp. *Anthriscus* sp.
	***n*-hexane–*n*-propanol (99.5:0.5)**	+ 5° atmosphere of N_2	
ethanol PE ethanol–Ac	**benzene–PE (2:1)** **PE–96 % ethanol (14:1)** PE benzene–PE (3:1) 1st dim.: **benzene–PE (3:1)** 2nd dim.: **PE–96 % ethanol (14:1)**		
a piece of tissue is squeezed on the starting point acetone extract or PE extract	**PE–Ac–benzene (8.5:1:0.5)**	time: 1 h S.F.–S.P.: 18 cm	*Solanum lycopersicum* *Vitis vinifera* *Hordeum* sp. *Zea mays*
chloroform	**petrol (b.p. 100–140°)–benzene–chloroform–Ac–isopropanol (50:35:10:0.5:0.17)**	time: 24 h vacuum	*Avena* *Veratrum* *Buddleia* *Hedera* *Parthenocissus* *Corylus*
	Me–PE (?) PE (b.p. < 80°) Me		
acetone extract	**toluene–carbon disulfide (10:3)** **toluene–carbon tetrachloride (1:1)** **PE–xylene–Ac (10:1.5:1)** PE–toluene–Ac (10:1.5:1) benzene–glycerol–chloroform (5:3:1) petrol–ethylene glycol–Ac (5:3:1) petrol–ethylene glycol–Me (3:2:1) PE–benzene–dioxan (10:1.5:0.5) PE–carbon tetrachloride–Ac (10:1:1)	S.F.–S.P.: 20–25 cm	*Triticum*
PE	**PE–benzene–chloroform–isopropanol (89:5:5:1)** **PE–benzene–*n*-propanol (94:5:1)** **benzene–PE–Ac (10:2.5:2)** **hexane–*n*-propanol (99.5:0.5)** **PE–chloroform–*n*-propanol (3:1:0.01)** benzene–PE–Ac–methanol (10:2.5:1:0.25) PE–benzene–*n*-propanol (88:10:2) hexane–*n*-propanol (99:1) PE–*n*-propanol (99.5:0.5) PE–chloroform (3:1)	+ 5°	*Triticum vulgare* *Secale cereale* *Pelargonium zonale* *Malva neglecta* *Lamium album* *Urtica urens* *Parthenocissus tricuspidata*

TABLE

METHODS BASED ON THE PRINCIPLES AND "REVERSED-PHASE"

1	2	3	4	5
DOUIN[15,16], 1953, 1954	1	A	Whatman No. 4; Durieux 147 (25 × 3 cm)	moistened with water to 10% water content (8–15%)
SERCHI, MICHI AND RAPI[48], 1953	disc method		W 1 pressed filter paper	
SPITERI AND NUNEZ[56], 1952	1			impregnated with triglycerides, chloro- or bromonaphthalene
STRAIN[59], 1953	1	A	cellulose paper; Eaton-Dikeman 301, 0.075 or 0.125 cm thick; glass paper	(a) **adsorption on the surface of the glass or cellulose** without special treatment or dried at 100° (b) **separation on the surface of a fixed liquid:** moistened with water or with 10% solutions of polyhydroxy compounds (glycerol, sorbitol) in water, or moistened with glycerol or sorbitol in methanol, or with glycerol + glycine + urea, or glycerol + 10% methanol (c) **separation by partition between immiscible solvents** sprayed with 70, 80 or 90% methanol or dipped in these solutions and blotted (d) **reversed-phase method:** impregnated with 5% solution of vaseline

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6	7		8	9
PE	**methanol 100°** ethanol propanol butanol monochlorobenzene chloroform benzene PE carbon disulfide carbon tetrachloride xylene		time: 20–30 min S.F.–S.P.: 12–15 cm spot distribution not influenced by temperature within the range 5–20° atmosphere saturated with H_2O	*Carex remota* *Tilia silvestris* *Anabaena cycadeae* *Bryales*
	stationary phase	*mobile phase*		
petrol–benzene–Me (9:1:3)	**H_2O**	**Ac** **petrol–Ac (7:3)** **Ac–petrol (9:1)** **benzene–chloroform (7:3)**		*Spinacia oleracea*
	ethyl ether	**Ac–petrol (9:1)** **Ac–petrol–H_2O (7:3:1)**		
	H_2O	chloroform benzene petrol butanol petrol–benzene saturated with ethanol (9:1) petrol–benzene (9:1) Ac–petrol–H_2O (7:3:1) petrol–benzene–Ac (7:1:2)		
	ethyl ether	ethanol–H_2O (4:1) alcohol (+ 3 drops of HCl)		
	ethyl acetate pyridine ligroin	chloroform benzene butanol		
	methanol **ethanol** **propanol**			
PE	(a) **PE–propanol (99.5:0.5)** PE		time: 20–40 min	various grasses (Poaceae)
	(b) **PE–propanol (99.5:0.5)** PE			
	(c) PE			
	(d) 80% methanol			

* For abbreviations and column headings, see Table 1.

TABLE

CHROMATOGRAPHIC

1	2	3	4	5
GRANGAUD AND GARCIA[23,24], 1952	1	A	Whatman No. 1 (50 × 15 cm)	
NUNEZ[40], 1954	1			impregnated with a mixture of triglycerides (0.5–4% olive oil in benzene), then dried at laboratory temperature
SAPOZHNIKOV, BRONSHTEIN-POPOVA, KRASOVSKAYA AND MAYEVSKAYA[46], 1956	1	A	chromatographic paper manufactured in U.R.S.S. (18 × 16 cm)	

reversed as follows (beginning from the original spot): carotenes–chlorophyll a–chlorophyll b–lutein–zeaxanthin–violaxanthin–neoxanthin[59].

4. FACTORS AFFECTING THE DISTRIBUTION OF SPOTS ON PAPER

The sequence of spots of chlorophylls and carotenoids on paper is determined first of all by the composition of the developing solvent mixture. The ratio of polar to nonpolar solvents in the mixture seems to be of the greatest importance. (The solvents may be arranged according to their polarity-index C:O—see, *e.g.*, PROCHÁZKA[42].) In pure nonpolar solvents only nonpolar carotenes are separated from the original spot, the other pigments either not moving at all or proceeding rather slowly. In solvent mixtures containing just a small quantity of polar solvents, the movement of chlorophylls, xanthophylls and pheophytins becomes greater. The relation between perfect separation and the ratio of polar and nonpolar solvents is clearly evident, especially in mixtures containing strongly polar solvents (*e.g.* alcohols); by increasing the percentage of strongly polar solvent, the "R_F" values of chlorophylls and xanthophylls increase. With mixtures containing larger quantities of strongly polar solvents as well as with pure polar solvents (acetone[5,8], ethanol[5], butanol[5]) all pigments move with the solvent front and are not separated at all.

Good evidence for this general statement may be found in the literature: *e.g.*, CHIBA AND NOGUCHI[14] have reported that the "R_F" of chlorophyll a is equal to 0.07–0.11 after 15 min development with pure toluene, but with the mixture toluene–95% ethanol (200:1) the "R_F" is 0.47. When using nonpolar petroleum ether only carotenes are separated[8,45], but xanthophylls and chlorophylls move with the mixture of petroleum ether and benzene (1:3 or 1:2)[45,46], which is further away from the nonpolar end in the table of solvents cited above[42] than petroleum ether. The influence of the degree of polarity of the solvent used is evident from the figure in the paper of MÁRKUS[38]. The "R_F" of chlorophyll a (similar differences in "R_F" values can be

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5*

SEPARATION OF CAROTENOIDS

6	7	8	9
PE	PE	atmosphere saturated with watervapours	*Tecoma radicans*
	ethanol–pyridine ethanol methanol propanol pyridine		*Solanum lycopersicum*
ethanol–acetone (1:3)	**benzene–PE (3:1)** **benzene–PE–96% ethanol (18:6:1)**	45 min	

* For abbreviations and column headings, see Table 1.

observed with chlorophyll b and xanthophylls) is *ca.* 0.03 when developing with pure petroleum ether, but it is 0.27 with petroleum ether–acetone (20:1), and 0.87 (!) with petroleum ether–methanol in the same ratio (methanol being a more polar solvent than acetone). When developing with nonpolar petrol (b.p. 60° to 70°) chlorophylls remain at the starting point, while an addition of 1 part of ethyl ether (semi-polar solvent) to 20 parts of petrol brings the "R_F" of chlorophyll a to *ca.* 0.06, and an addition of 1 part of acetone (more polar than ethyl ether) raises the "R_F" to 0.44. Similarly the "R_F" of chlorophylls is increased by adding 0.5% propanol to pure petroleum ether[59]. Smaller amounts of the more polar solvent in a mixture (0.25 volume parts of methanol) produce higher "R_F" values than greater quantities (1 part) of the somewhat less polar solvent (acetone), the proportion of other solvents remaining unchanged[8]. Replacing two parts of acetone in the mixture petrol–acetone–benzene by 1 part of the more polar solvent—methanol—raises the "R_F" value of chlorophyll a from 0.38 to 0.9[38]. According to ref. 46, after addition of the polar solvent—ethanol (mixture of benzene–petroleum ether–96% ethanol in the ratio 3:1:0.33) the rate of movement of chlorophylls and xanthophylls is higher than with the mixture benzene–petroleum ether (3:1). When 0.03 volume parts of *n*-butanol, which is less polar than acetone, are added to the mixture petrol (b.p. 60–70°)–acetone (20:1) the "R_F" value of chlorophyll a decreases from 0.44 to 0.22[38].

The observations cited above seem to support the idea that a perfect separation of chlorophylls and xanthophylls would require suitable relative quantities of polar solvents in a mixture. Some results obtained by ASAMI[5] may be quoted against this opinion. He succeeded in resolving pigments with relatively nonpolar solvents—toluene or tetrachloromethane (or with xylene or benzene); the "R_F" values were essentially the same regardless of the fact whether these solvents were anhydrous or saturated with water. Some results of MÁRKUS[38] are also in opposition to the opinion mentioned above: on developing with the mixture petrol–chloroform (20:1), the "R_F" of chlorophyll a was found to be 0.15, while when chloroform was replaced by the more

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polar solvent ethyl ether, the "R_F" was only *ca.* 0.06. Contrary to expectation, addition of 1 part of toluene to the mixture petroleum ether–acetone (20:1) raised the "R_F" values of chlorophyll from 0.27 to 0.4. The situation therefore seems to be still very complicated and it would be rather difficult to draw definite conclusions from all these results because they are not very comparable.

Determination of constant "R_F" values of plastid pigments is impossible, for these values depend on the quantities of compounds analysed[5,7,8,14,19,27,28,32,35,39,49,50,52], on the duration of development[5,13,15,16], and, in the case of impregnated paper, on the amount of compound impregnated in the paper[40]. With increasing concentration of pigments in the sample analyzed, the "R_F" values increase, while they decrease with increasing quantity of impregnation compound, and with increase in developing time. The spots of chlorophylls usually have long diffusion "tails", sometimes observable only in U.V. light. The use of samples containing approximately the same quantities of pigments has been recommended in order to obtain comparable chromatograms.

When extracts from different plant species are chromatographed using one method, the sequence of spots and degree of separation may be different. This phenomenon is sometimes imputed to the presence of colourless liposoluble compounds in the extract. In some experiments resolution became more regular if purified extracts were used or if mixtures of pure isolated pigments were analysed[5,7,15,49].

The separation is also affected by the quality of paper[38], although according to some authors[45,50] the usual filter paper is quite adequate. In most cases moderately fast running papers have frequently been used (Whatman No. 1, Schleicher and Schüll 2043 b).

Some authors recommend transferring pigments to petroleum ether prior to spotting on paper. This technique is supposed to remove the greater part of undesirable material, especially hydrophilic pigments, from the extract[15,16,30,36,49,59]. In most papers, however, it is assumed that a concentrated extract can be spotted directly on paper. Some authors[35,52] have even squeezed a piece of plant tissue on the starting point. Usually, the sample is applied as a little round spot or as a streak[15,23,27,28,46,54,57].

The decomposition of pigments during developing is sometimes prevented by keeping the temperature between $+1°$ and $+5°$ [21,32,49,57], by impregnating the paper with sucrose[21,49,57], or by making use of an inert atmosphere in the chamber[21,57]. The chamber must always be kept dark.

5. USE OF THE METHOD

The method described has been used (in the communications mentioned above and in other papers) for qualitative and quantitative determination of pigments in lower and higher plants[17,18,30], for identification of pigments in those plant tissues where their presence is unusual, *e.g.* in bark and xylem[25,26], for studying preparations of plant material before extraction[6], for isolation of small quantities of pigments[12], for studying various factors affecting pigment synthesis in plants[27,29,47,50,51,54], for observation of genetic effects of X-rays[43,44], for genetic survey of varieties of rice[32,33], for analysis of

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chlorophyllase activity[22,53,54,61], for studying the decomposition of pigments in sediments and muds[1], for determining the extent of smoke damage in forestry[39], as well as in food chemistry[19,20] and pharmacology[41,48].

Paper chromatography has been used in rare cases to separate the crystalline chlorophyll–lipoprotein complex into two components[2,13]. These components were separated by the ascending technique, on Whatman No. 1[2] or Toyo No. 50 paper[13], a mixture of picoline and water (1:1) being used in both cases.

Methods for separating various porphyrin pigments, some of which are precursors of chlorophylls or their degradation products are not discussed in this review.

6. CONCLUSION

More than 50 papers dealing with paper chromatography of plastid pigments have been published up to the present. These methods are summarized in the text or in the tables. A really critical comparison of the methods described in the literature cannot be presented in this review. Such a comparison would require a special study. All the techniques quoted above should be tested using uniform material and under constant conditions.

In spite of the lack of methods for the complete separation of all plastid pigments in a pure state, a number of techniques examined up to the present time have been used for solving diverse problems. The rapid advances that are being made in the development of this analytical procedure hold out promise that it will be very soon perfected.

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CHROMATOGRAPHIC IDENTIFICATION OF ANTHOCYANIN PIGMENTS

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I. INTRODUCTION

Chromatography is well recognised to-day as an indispensable technique in the study of all kinds of plant pigments. Only in the last ten years, however, has it been applied to the separation of the water-soluble pigments, such as the anthocyanins. Attempts to separate them by absorption chromatography on columns, a technique which had so successfully been applied to the carotenoid pigments[1], did not meet with much success[2,3]. It was not until the introduction of paper partition chromatography into this field by BATE-SMITH in 1948[4,5] that satisfactory chromatographic methods for separating the anthocyanins and related flavonoid pigments became available. Since then, paper chromatography has been used in the identification of individual pigments, especially the minor components, in complex mixtures of flavonoid compounds, such as are found in the flowers of *Antirrhinum majus*[6], *Dahlia variabilis*[7] and in many other plant extracts. As a result of these and other investigations (*e.g.*[8]) the R_F values of all the main classes of flavonoid compounds, including the anthocyanins, became available and some general reviews of these data have already appeared[9–11].

This review is concerned only with the chromatography of the anthocyanins, the compounds responsible for most of the red, purple and blue pigmentation in plants[12]. They are more difficult to characterise by conventional methods than the related flavones and the well known colour and distribution tests[13] are limited in their scope. For these reasons, chromatographic methods are of especial importance for characterising anthocyanins. Their value may be illustrated by the fact that the recent discoveries in plants of two new anthocyanin aglycones, *i.e.* anthocyanidins[14,15], and several new classes of anthocyanin[16,17] were only made possible by means of paper chromatography. It has also been shown in this laboratory[17,18] and elsewhere[19,20] that unknown pigments can be quickly and economically identified on a micro-scale by the aid of chromatographic techniques.

The paper chromatography of the anthocyanins will first be reviewed and some previously unpublished R_F values will be given and discussed. A description of the value and limitations of the chromatographic approach to anthocyanin identification will form the main part of this article. Finally, some examples will be given of the use that has already been made of chromatographic methods in studying the occurrence and distribution of anthocyanins in plants.

Before closing this introduction, it is necessary to consider what is involved in

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characterising anthocyanins. The main facts of their chemistry are now well established (for review, see, *e.g.* WAWZONEK[21]). They occur in nature in combination with sugars (as glycosides) and occasionally also in association with organic acids (as acylated or "complex" glycosides). There are only a few different anthocyanidins formed on acid hydrolysis of anthocyanins and all have the same basic chemical structure (see Fig. 1). In addition, the positions at which sugars may be attached to

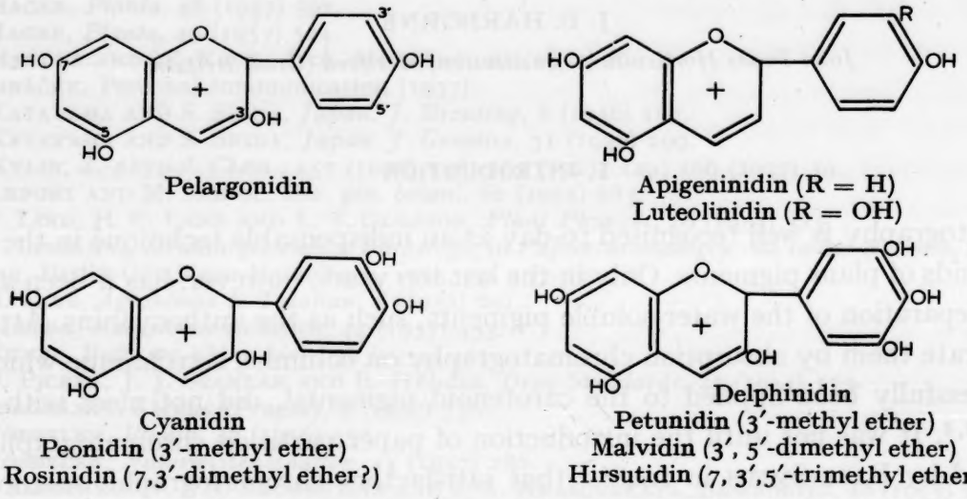

Fig. 1. The naturally occurring anthocyanidins.

hydroxyl groups in the anthocyanin molecule are restricted and residues are only normally found in the 3-position or in the 3- and 5-positions. The characterisation of an unknown pigment thus depends on identifying the anthocyanidin produced on hydrolysis and on determining the nature, position of attachment and number of sugars present. In addition, examination for the presence of an acyl component must be made and if present, the organic acid concerned must be identified.

It will be shown here that chromatography can be used at each step in the identification of anthocyanins. It must however be emphasised that chromatographic methods should be combined with other techniques for the complete structural determination of anthocyanins. For example, spectral methods are important[3,22] and can be used most profitably in conjunction with chromatographic studies. Finally, it must be stressed that considerable experience is necessary for interpreting chromatographic data, which must be related to similar data obtained from reference compounds.

2. PAPER CHROMATOGRAPHY OF ANTHOCYANINS

The use of paper chromatography in the study of anthocyanins is a logical extension of the tests devised by ROBINSON AND ROBINSON[13] for distinguishing different glycosidic classes according to their distribution between water and amyl alcohol. BATE-SMITH[4,5] first showed that anthocyanins could be readily separated on filter paper developed with butyl alcohol–acetic acid–water and he published R_F values for most of the anthocyanins that were known at the time and that had been synthesised in the laboratory. Other workers[9,10,23] have since published R_F values for these and

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other anthocyanins and several more solvent systems have been employed. The general conditions for the paper chromatography of the anthocyanins are now well appreciated and are as follows.

Extraction

Extracts of plant organs containing pigment can be examined directly by paper chromatography. The solvents used for extraction are methanol or water containing 1% hydrochloric acid. Methanol is generally to be preferred since the extracts are more easily concentrated prior to their application to paper. This is important when extracts are used of plant organs which contain only small amounts of anthocyanin. Leaf extracts are normally washed with petroleum ether to remove chlorophyll, before being concentrated. The R_F values of anthocyanins, present in crude plant extracts, are not completely reliable since the position and number of the spots may be affected by the presence of other components, especially flavonol glycosides. Therefore, in order to obtain accurate R_F values it is essential to purify anthocyanins in plant extracts either by the more usual methods (*cf.*[21]) or by chromatography on thick Whatman paper (see section 3, p. 215). Anthocyanins so obtained are best dissolved in 1% methanolic hydrochloric acid before being applied to paper.

Filter paper

Whatman No. 1 paper is commonly employed for the chromatography of anthocyanins. All R_F values quoted in this review were measured on this paper. The corresponding grades used by German and Japanese workers are Schleicher and Schüll 2043b MGL and Toyo No. 50 respectively.

Solvent systems

The solvent systems used are listed in Table 1. Because anthocyanins are cations and are only stable at acid pH, chromatography is normally carried out in solvent systems

TABLE 1

SOLVENT SYSTEMS FOR THE CHROMATOGRAPHY OF ANTHOCYANINS

Abbreviation	*Composition*	*Proportions (v/v)*	*Layer used*
BAW	*n*-butanol–acetic acid–water	4:1:5*	top**
BuHCl	*n*-butanol–2 *N* hydrochloric acid	1:1	top**, ***
—	*m*-cresol–acetic acid–water	50:2:48	top
isoPr.	isopropanol–2 *N* hydrochloric acid	1:1	miscible
PhOH	phenol–water	saturated	bottom
—	acetone–0.1 *N* hydrochloric acid	1:3	miscible
—	ethyl acetate–formic acid–water	8:2:3	top
1% HCl	water–12 *N* hydrochloric acid	97:3	miscible
HAc–HCl	water–acetic acid–12 *N* hydrochloric acid	82:15:3*	miscible
30% HAc	water–acetic acid	70:30*	miscible

* Other proportions of these mixtures can also be used.

** The time of use after mixing is important and affects the R_F value.

*** BuHCl should be left for 24 h before use and the paper must also be equilibrated in a tank containing the lower aqueous phase of BuHCl for 24 h.

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containing acid. If solvents do not contain mineral acid, *e.g.* BAW, it is important that sufficient hydrochloric acid is present in the original extract to keep it in chloride form as it travels down the paper[4]. As will be seen in Table 1, the solvent systems are mainly of two types, based either on an alcohol (*e.g. n*-butanol) or on water. The relative merits of some of these systems are discussed by ABE AND HAYASHI[23]. The author has found BAW, BuHCl, 1% HCl and HAc–HCl to be a useful selection of solvents, each of which gives consistent results. The only solvent system in which it is essential to equilibrate the paper before development in the aqueous phase, is BuHCl. Special care must also be taken with BAW, since the length of time this solvent mixture is allowed to stand before use can affect the R_F values considerably. For measuring standard R_F values, an *n*-butanol–acetic acid–water mixture which has been aged for three days before use, is recommended[5]. For general use, a fresh mixture of these solvents is quite satisfactory and such mixtures have been used routinely in this laboratory.

Chromatograms are normally developed with these solvents by descent, but it has been claimed[24] that more reliable results are obtained with BAW if it is allowed to ascend the paper. Suitable solvent pairs for two-dimensional paper chromatography are BAW and 15% HAc or BuHCl and 1% HCl. Anthocyanins tend to fade and form large diffuse spots when chromatographed in two directions, so that this method has not been greatly used. Circular paper chromatography has also been used with anthocyanins[25].

Visualisation

Different anthocyanins appear as different coloured spots on chromatograms (see Table 2) when seen in daylight and characteristically change colour on fuming the

TABLE 2

R_F VALUES AND COLOURS OF PELARGONIDIN GLYCOSIDES

*Pelargonidin glycoside**	*R_F values in*				*Visible*	*Colours in ultra-violet*
	BAW	*BuHCl*	*1% HCl*	*HAc–HCl*		
3-monoglucoside	0.44	0.38	0.14	0.35	orange-red	dull red
3-monogalactoside	0.39	0.37	0.13	0.33		
3-rhamnoglucoside	0.37	0.30	0.22	0.44		
3-diglucoside I**	0.36	0.26	0.50	0.62	orange-red	dull red
3-diglucoside II***	0.30	0.17	0.21	0.47		
3-triglucoside	0.25	0.10	0.35	0.52	orange-red	dull red
3,5-diglucoside	0.31	0.14	0.23	0.45		
3-rhamnoglucosido-5-glucoside	0.29	0.13	0.40	0.58	orange	fluorescent yellow
3-diglucosido (1)-5-glucoside	0.25	0.10	0.60	0.68		
3-diglucosido (1)-7(or 4′)-glucoside	0.18	0.04	0.73	0.73	orange-yellow	dull orange red

* Sources are given in refs. [17,22].
** Isolated from *Papaver rhoeas*.
*** Isolated from *Primula sinensis*, possibly the 3-gentiobioside.

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paper with ammonia[4]. Examination under ultra-violet light is also of value, since differences in ability to fluoresce provide a means to their identification[17,23]. Pelargonidin and peonidin 3,5- and 5-glycosides appear as intensely fluorescent yellow and pink spots, while the corresponding 3-glycosides are non-fluorescent. Similar, but less striking, differences have been noted with glycosides of the other anthocyanidins. The colours produced by anthocyanins after chromatograms developed in BAW or 15% HAc have been sprayed with a 5% ethanolic solution of aluminium chloride[26], or 2% aqueous ferric chloride[27] have also been noted. These sprays distinguish derivatives of cyanidin, delphinidin and petunidin (positive colour change) from those of the other anthocyanidins (no colour change).

R_F values

The R_F values of anthocyanins as determined in this laboratory are given in Tables 2, 3 and 4. They have been chosen to illustrate the relationship that exists between chemical structure and R_F value in this series, and at the same time, to provide a useful collection of data for identification purposes. The results obtained

TABLE 3

R_F VALUES AND COLOURS OF CYANIDIN AND PEONIDIN GLYCOSIDES

Glycoside	*R_F values in*				*Visible*	*Colours in ultra-violet*
	BAW	*BuHCl*	*1% HCl*	*HAc–HCl*		
Cyanidin:						
3-monoglucoside	0.38	0.25	0.07	0.26	magenta	dull
3-monogalactoside	0.37	0.24	0.07	0.26		magenta
3-rhamnoglucoside	0.37	0.25	0.19	0.43		
3-xyloglucoside	0.36	0.24	0.24	0.51	magenta	dull
3-diglucoside	0.33	0.22	0.34	0.61		magenta
3,5-diglucoside	0.28	0.06	0.16	0.40	magenta	bright
3-rhamnoglucosido-5-glucoside	0.25	0.08	0.36	0.59		red
Peonidin:						
3-monoglucoside	0.41	0.30	0.09	0.33	pink	dull pink
3,5-diglucoside	0.31	0.10	0.17	0.44	pink	fluorescent
3-rhamnoglucosido-5-glucoside	0.29	0.12	0.37	0.60		rose

here are in general agreement with values obtained by other workers[5,23] and include figures for a number of anthocyanins not previously available. No attempt has been made to run all chromatograms under standard conditions, but the results given are strictly comparable and are typical values from a large number of determinations.

Tables 2, 3 and 4 give the R_F values and colours of the most frequent anthocyanins, including the 3-monoglucosides and 3,5-diglucosides of all the common anthocyanidins. Table 2, giving the R_F values of ten glycosides of pelargonidin, shows the range of R_F values that can be obtained for a series of different glycosides of the same anthocyanidin. It is also to be noted that no two glycosides have exactly

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TABLE 4

R_F VALUES AND COLOURS OF DELPHINIDIN, PETUNIDIN AND MALVIDIN GLYCOSIDES

Glycoside	R_F values in				Visible	Colours in ultra-violet
	BAW	BuHCl	1% HCl	HAc–HCl		
Delphinidin:						
3-monoglucoside	0.26	0.11	0.03	0.18	purple	dull purple
3-rhamnoglucoside	0.30	0.15	0.11	0.37	purple	dull purple
3,5-diglucoside	0.15	0.03	0.08	0.32	purple	purple
Petunidin:						
3-monoglucoside	0.35	0.14	0.04	0.22	purple	dull purple
3-rhamnoglucoside	0.35	0.16	0.13	0.42	purple	dull purple
3,5-diglucoside	0.24	0.04	0.08	0.32	purple	bright
3-rhamnoglucosido-5-glucoside	0.23	0.06	0.37	0.61		purple
Malvidin:						
3-monoglucoside	0.38	0.15	0.06	0.29	mauve	dull mauve
3,5-diglucoside	0.31	0.03	0.13	0.42	mauve	fluorescent
3-rhamnoglucosido-5-glucoside	0.30	0.05	0.40	0.63		cerise

the same R_F values. R_F values in BAW and 1% HCl of three different glycosides of the six main anthocyanidins are plotted graphically in Fig. 2, after the manner of ABE AND HAYASHI[23], to illustrate the relationship between structure and R_F value. The glycosides are the 3-monoglucosides (3G), the 3,5-diglucosides (3G5G) and the 3-rhamnoglucosido-5-glucosides (3RG5G). This latter group of glycosides has only recently been discovered[16,17] and no R_F values have previously been given for its members.

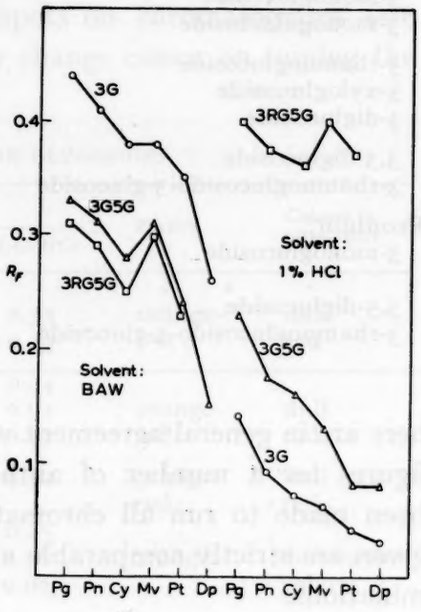

Fig. 2. Graph of the R_F values of the 3-glucosides (3G), 3,5-diglucosides (3G5G) and 3-rhamnoglucosido-5-glucosides (3RG5G) of pelargonidin (Pg), peonidin (Pn), cyanidin (Cy), malvidin (Mv), petunidin (Pt) and delphinidin (Dp).

R_F value and structure

Regular relationships between the R_F value and structure of an anthocyanin have been noted by BATE-SMITH AND WESTALL[28], by ABE AND HAYASHI[23] and by REZNIK[10]. Their results have been amplified by studies in this laboratory and the general conclusions have been shown to hold good for flavones and other polyphenols (see, *e.g.*[28,29]). The results are best considered in relationship to particular structural modifications.

(1) *Hydroxylation.* The greater the number of hydroxyl groups present in the anthocyanidin molecule, the lower the R_F value is, in both alcoholic

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(BuHCl and BAW) and aqueous (1% HCl and HAc–HCl) solvents. Thus corresponding glycosides of pelargonidin, cyanidin and delphinidin can always be placed in decreasing order of R_F value.

(2) *Methylation* reverses the effect of hydroxylation, so that the greater the number of methoxyl groups, the higher is the R_F value, again in both types of solvent (see Table 4 especially). The increase in R_F value brought about by methylation is rather less than the decrease caused by hydroxylation. Thus the same glycosides of cyanidin and malvidin have rather similar R_F values (Tables 3 and 4). This effect is observed not only of methylation in the 3′- and 5′-positions in the anthocyanin molecule, but also of methylation in the 7-position, as shown by the figures for hirsutin[28] and rosinin[14].

(3) *Glycosidation.* There is a direct relationship between R_F value and the number of sugar residues which is quite independent of the nature of the anthocyanidin. In aqueous solvents, the effect of glycosidation is to increase R_F values; in BAW or BuHCl, the exact reverse is true (see Fig. 2). This is well illustrated by the figures for the pelargonidin 3-mono-, di- and triglucosides which occur in *Primula sinensis*[17] (Table 2). It is also to be noted that this simple relationship is complicated if sugar residues are attached to more than one position in the molecule and also depends on the nature of the sugar–sugar linkage in the case of the 3-biosides[17].

(4) *Acylation* causes an increase in R_F value in solvents based on *n*-butanol, but lowers the R_F value in aqueous solvents. The effect of acylation on R_F value is the reverse of that shown by glycosidation. The R_F values of acylated anthocyanins will be further discussed under section 6, p. 220.

3. SEPARATION AND PURIFICATION

(*a*) *By paper chromatography*

The first step in the detailed examination of an anthocyanin pigment is that of isolating it in a pure state. It must be separated from other compounds which are extracted from the plant at the same time, namely other anthocyanins, flavone glycosides (co-pigments) and water-soluble substances such as free sugars. The usual chemical methods of isolation are often tedious and wasteful (*cf.*[21]) and do not always yield a pure compound (see, *e.g.* SCOTT-MONCRIEFF[30]). Paper chromatography provides a convenient and rapid alternative means of preparing pure anthocyanins on a small scale without incurring much loss.

The general procedure is similar to that used for flavone glycosides[7,31] and has been described in outline already[17]. An extract of the plant material with methanolic hydrochloric acid is concentrated and applied directly to several sheets of thick filter paper (Whatman No. 3 or 3MM) and chromatography is carried out in BAW, BuHCl or, less frequently, 1% HCl. The anthocyanins appear as clear discrete coloured bands, which are then cut out and the paper eluted with methanol containing hydrochloric acid or acetic acid and the process is repeated twice with the same or a different solvent mixture. It is important to carry out these operations quickly, since solutions

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of anthocyanins tend to fade on exposure to light. Anthocyanin bands, when cut into strips five inches long, can be eluted with methanol in a very short time (1–2 hours), especially if they are cut from chromatograns which are only just dry. Pigments which have been left on filter paper strips for several weeks cannot always be completely recovered by elution, due to some irreversible adsorption having taken place. Anthocyanins which occur in very complex mixtures of flavonoids may require further purification on paper and some acylated pigments of closely related structure do not separate well under these conditions (see section 6).

Using these methods, a large number of anthocyanins have been successfully purified in this laboratory. In some instances, sufficient material has been obtained from paper chromatograms to yield the pigment in crystalline form. As many as eight different anthocyanins have been separated from a single plant extract, that of the stems of certain *Primula sinensis* genotypes[32]. Indeed the method is particularly recommended for separating the anthocyanins in plants which contain several such pigments. It has already been used successfully for this purpose with the pigments of grapes[20], potato tubers[15] and of many garden flowers.

(*b*) *By column chromatography*

Column chromatography provides a method of separating anthocyanins on a preparative scale, but the high degree of resolution that can be achieved on filter paper has not yet been duplicated on a column. Mixtures of two or three anthocyanins have been separated by this means[2,3,33,34], but the method is only partially successful with plant extracts containing more than three pigments[35].

Difficulty has been experienced in finding a suitable adsorbent for anthocyanins. They are so strongly adsorbed on to columns of basic salts, such as alumina, that it is not easy to elute them from the column. The choice of eluting solvent is also restricted to those containing *n*-butanol or a similar alcohol and aqueous or methanolic hydrochloric acid. The use of alumina and a specially chosen grade of calcium sulphate by KARRER AND STRONG[2] and KARRER AND WEBER[33] for separating mixtures of two anthocyanins has not found favour in the more recent work of LI AND WAGENKNECHT[3]. These latter authors successfully separated the 3-rhamnoglucoside and 3-diglucoside of cyanidin, present in sour cherries, on silicic acid columns by elution with BAW.

However, the most popular adsorbent for separating anthocyanins is cellulose, but it is clear that the success of the operation is dependent on the grade of cellulose powder[36] and the method of packing[34] employed. ENDO[36] separated the six anthocyanins present in the flowers of *Viola tricolor maxima* on cellulose, but only one particular batch of the powder was really successful. After separation, the column was extruded and the bands cut out and eluted with methanol. CHANDLER AND HARPER[35] in separating six pigments present in blackcurrants on cellulose, eluted the pigments directly from the column. The faster moving anthocyanins had however to be further purified by lead precipitation.

Column chromatography is also used for separating anthocyanins from other components of plant extracts in a preliminary purification in which no separation of

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individual pigments is attempted. The anthocyanins are strongly adsorbed on to the column, which can then be washed thoroughly with water to remove impurities. The pigments are then eluted from the column with methanolic hydrochloric acid. Columns of paper[37], lead hydroxide[38] and magnesol–celite (5:1) mixtures[39] have been used for this purpose.

4. CHROMATOGRAPHIC IDENTIFICATION OF ANTHOCYANIDINS AND SUGARS

On acid hydrolysis, anthocyanins give rise to anthocyanidins (aglycones) and sugars. The identification of the aglycone and sugar moieties of unknown pigments is most satisfactorily accomplished by chromatographic comparison on paper with standard markers. The type of procedure to be used has been described by HARBORNE AND SHERRATT[18] and NORDSTRÖM[19] and works well with the amounts of anthocyanin that can be obtained after separation and purification on paper.

The choice of solvent for the chromatography of anthocyanidins is limited by the fact that they are even less stable than the anthocyanins to pH and light. For example, anthocyanidins tend to fade when chromatographed in BAW. For this reason satisfactory solvents for anthocyanidins usually contain mineral acid and the "Forestal" solvent (acetic acid–conc. HCl–water, 30:3:10 v/v) has been widely used[40]. A similar type of solvent system (formic acid–conc. HCl–water, 5:2:3 v/v) is also useful, as with this mixture, anthocyanidins are particularly well separated from unhydrolysed anthocyanin, the presence of which can cause confusion on "Forestal" chromatograms. BAW may be used for anthocyanidins, if the filter paper used is previously washed with dilute hydrochloric acid. A number of other solvent systems have been suggested for anthocyanidins (*e.g.* BuHCl and *m*-cresol–acetic acid–conc. HCl, 1:1:1 v/v) but none of these can be considered to be completely satisfactory due to tailing of the pigment spots and the presence of "chloride" fronts on chromatograms developed with these mixtures. Anthocyanidins have also been separated on a larger scale by column chromatography on cellulose[41] or silicic acid[42].

The regular relationship that exists between R_F value and chemical structure

TABLE 5

R_F VALUES AND COLOURS OF ANTHOCYANIDINS

	R_F values in			*Visible*	*Colours in ultra-violet*
	Forestal	*Formic*	*BAW**		
Hirsutidin	0.78	0.36	0.66	purple	mauve
Malvidin	0.60	0.27	0.58	purple	mauve
Petunidin	0.46	0.20	0.52	purple	mauve
Delphinidin	0.32	0.13	0.42	purple	mauve
Rosinidin	0.76	0.39	—	magenta	pink
Peonidin	0.63	0.30	0.71	magenta	pink
Cyanidin	0.49	0.22	0.68	magenta	pink
Pelargonidin	0.68	0.33	0.80	red	orange-red
Luteolinidin	0.61	0.35	0.56	orange	red-brown
Apigeninidin	0.75	0.44	0.74	yellow	yellow-brown

* Run on acid-washed paper.

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with the anthocyanins is also found in the anthocyanidin series. The distance travelled in "Forestal" solvent is directly related to the number of free phenolic hydroxyl groups and the most highly hydroxylated anthocyanidin, delphinidin, has the lowest R_F value (Table 5). On the other hand, methylation of the hydroxyl groups reverses this effect in a regular way, as can be seen in the series delphinidin, petunidin, malvidin and hirsutidin.

The sugars produced from anthocyanins on acid hydrolysis are identified by the usual chromatographic procedures (for review, see LEDERER AND LEDERER[43]).

The use of either di-*n*-octylmethylamine in chloroform[18] or of ion exchange resins[7] is recommended for the removal of mineral acid from the sugar hydrolysates before chromatography. It is important to distinguish between anthocyanins containing glucose and those containing galactose, so that at least one chromatogram should be developed with a suitable solvent system for 2–3 days, in order to separate these two sugars. At the same time, disaccharides, which are occasionally present in anthocyanin hydrolysates, are more easily detected. When two isomeric 3-diglycosides of the same anthocyanidin are known, for example of pelargonidin[17], the identification of the disaccharides found in this way becomes important.

One difficulty has arisen in this method peculiar to anthocyanins which have been purified by paper chromatography. It has been found[17] that arabinose is produced as an artifact by the action of mineral acid in the solvent system on filter paper. This arabinose then interferes with the normal detection of the sugars and gives misleading results. Other workers (NORDSTRÖM[19], ASEN, SIEGELMAN AND STUART[44]) have also been misled by the presence of arabinose in their sugar hydrolysates arising in this manner[17,37]. The production of arabinose can be avoided by carefully replacing the mineral acid used during the purification process by acetic acid. It has also been found advisable to wash the filter paper before use with dilute acetic acid to remove any soluble impurities.

Anthocyanins purified for sugar analysis should not be contaminated with free sugars or other plant glycosides. Free sugars are readily removed by routinely carrying out chromatography in an aqueous solvent (*e.g.* 15% HAc) during purification. Other plant glycosides are normally removed by using two or more solvent systems during purification, but it is not always possible to be certain that the anthocyanin is finally free from such contamination. Indeed, in two or three instances in this laboratory, incorrect sugar analyses have been attributed to this cause. The remedy is to repeat the sugar analysis on pigment which has been purified in a different set of solvent systems or in the same solvent systems in a different order. It has now become standard practice to do this in all cases where more than one sugar is detected. It should also be noted that anthocyanins which have been purified by column chromatography may be contaminated by free sugars[34,45].

5. DETERMINATION OF THE POSITION AND NUMBER OF SUGAR RESIDUES

The next step in the characterisation of an unknown anthocyanin, once its anthocyanidin and sugars have been identified, is to determine the position and number of

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these sugar residues or to find out to which glycosidic "class" the pigment belongs. Paper chromatography provides an excellent method of classifying anthocyanins in this way. Indeed, as a result of using this method, a number of new glycosidic classes have been found[16,17] and the strict classification of anthocyanins into the four "classes" described by the ROBINSONS[13] can no longer be made, since there are now at least ten such "classes" known. It is preferable to describe anthocyanins according to the actual sugars that are present, rather than to use the vaguer terms of the ROBINSONS' terminology, *i.e.* 3-monoside, 3-bioside, etc.

An unknown anthocyanin can be characterised by a careful comparison of its R_F values in several solvent systems with values of well characterised glycosides of the same anthocyanidin. If the unknown compound has the same R_F values as a well characterised glycoside, co-chromatography will confirm the identification. Even if it is not chromatographically identical with any of the known pigments, these data should provide a clear indication of the number of sugar residues present.

Anthocyanins can be further characterised by studying on paper chromatograms the simpler glycosides produced during acid[17,23] or enzymic hydrolysis[46]. The number

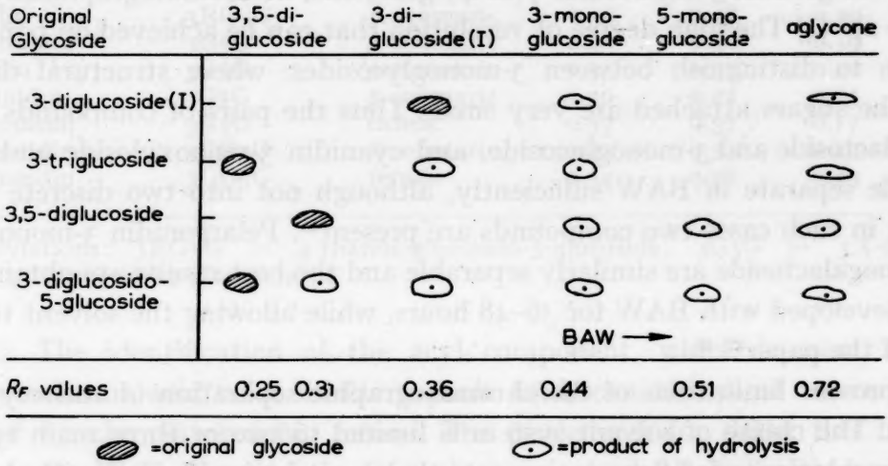

Fig. 3. A chromatogram of the products of controlled acid hydrolysis of some pelargonidin glucosides.

and identity of these simpler glycosides provide a valuable clue to the number and position of the sugar residues in the original glycoside. Thus 3-diglucosides and 3,5-diglucosides are distinguished by the fact that they give one and two simpler glycosides respectively during acid hydrolysis. Anthocyanins containing three sugar residues give two or four simpler glycosides in this way[16,17]. The individual components in the mixture of pigments present when acid hydrolysis is stopped before completion, can be separated and identified on paper chromatograms in the usual manner. The method works particularly well with derivatives of pelargonidin, as can be seen in Fig. 3, which illustrates the general principles of this approach. Alternative solvent systems for separating the glycoside mixtures are BuHCl and 1% HCl.

Identification by the above means depends on having available a number of pigments of known structure. Many of these can easily be obtained directly from plant

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sources, as described in the literature[13,21]. The sources of the new pigments isolated in this laboratory are described elsewhere[22]. The author was also fortunate in having a number of anthocyanins both natural and synthetic, which were left over from earlier work at this Institution. If no such specimens are available, it is advisable to check carefully the identity of plant material used for preparing authentic "markers", especially as different "varieties" of the same plant may differ in their pigment content. In addition, some of the earlier pigment identifications have had to be modified by later work[47].

The accuracy of chromatographic identifications made in the above manner depends on the following factors. (1) No exceptions to the very striking relationship between R_F value and number of sugar residues of the anthocyanin in several different solvent systems have yet been found. (2) Isomeric 3-diglucosides of pelargonidin have different chromatographic properties, so that it is possible to distinguish anthocyanins which only differ in the nature of the linkage between the two sugars that are attached. (3) Chromatographic methods will distinguish glycosides in which the sugars are attached in an unusual position. Thus the first 3,7- or 3,4′-glycoside to be found in nature[17], pelargonidin 3-diglucosido-7(or 4′)-glucoside, is chromatographically distinct (see Table 2). (4) The high degree of resolution that can be achieved on paper makes it possible to distinguish between 3-monoglycosides, where structural differences between the sugars attached are very small. Thus the pairs of compounds cyanidin 3-monogalactoside and 3-monoglucoside, and cyanidin 3-monoxyloside and 3-monoarabinoside separate in BAW sufficiently, although not into two discrete spots, to show that in such cases two compounds are present[45]. Pelargonidin 3-monoglucoside and 3-monogalactoside are similarly separable and the best results are obtained if the paper is developed with BAW for 36–48 hours, while allowing the solvent to drip off the end of the paper[17,48].

The present limitations of the chromatographic separation of anthocyanins are as follows. The choice of solvent system is limited to two or three main types. The excellent resolutions of different pigments that have been obtained with derivatives of pelargonidin and cyanidin, have not yet been obtained in the delphinidin series. Paper chromatography will not completely resolve acylated anthocyanins if they are of very similar structure.

6. ACYLATED ANTHOCYANINS

Before determining the glycosidic nature of an anthocyanin, it is important to know whether it is acylated or not, since acylation has a profound effect on R_F values. Information on this point can be obtained during the preliminary chromatographic studies. The most characteristic chromatographic feature of acylated pigments is the fact that when they are chromatographed in BuHCl, two spots are shown to be present, a main spot of high R_F value and a minor spot of low R_F. Even if the major component is separated chromatographically from the minor one, on rechromatography two spots will again appear. This point was first noted by DODDS AND LONG[49] and is due to the fact that the acyl linkage to the anthocyanin is more labile than the

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sugar linkages. It is therefore impossible to avoid some deacylation taking place during the preparation of an extract of any acylated pigment. On carrying out the deacylation with alkali and then reacidifying, the pigment appears as a single component of low R_F, the spot of high R_F having disappeared.

A simple test for acylation consists of comparing R_F values in different solvent systems of the pigment before and after alkaline treatment. If there is no change in R_F value, the pigment can be assumed to be free of labile acyl groups. All acylated pigments which have been examined so far do change their R_F value under these

TABLE 6

R_F VALUES OF ACYLATED ANTHOCYANINS

Acylated glycosides		*Acyl group*	*BAW*	*BuHCl*	*1% HCl*	*HAc–HCl*
Aglycone	*Sugars**					
Pelargonidin	3RG5G	*p*-coumaric	0.37	0.43	0.27	0.67
Cyanidin	3RG5G	*p*-coumaric	0.32	0.26	0.22	0.62
Peonidin	3RG5G	*p*-coumaric	0.34	0.31	0.22	0.62
Delphinidin	3RG5G	*p* coumaric	0.31	0.24	0.19	0.59
Petunidin	3RG5G	*p*-coumaric	0.32	0.26	0.19	0.59
Malvidin	3RG5G	*p*-coumaric	0.36	0.28	0.20	0.64
Pelargonidin	3G5G	*p*-coumaric	0.40	0.46	0.19	0.53
Cyanidin	3G5G	*p*-coumaric	0.35	0.34	0.11	0.43
Delphinidin	3G5G	*p*-coumaric	0.30	0.22	0.05	0.32
Pelargonidin	3G5G	caffeic	0.37	0.37	0.17	0.48
Pelargonidin	3GG5G	*p*-coumaric	0.34	0.34	0.49	0.73
Pelargonidin	3GG5G	ferulic	0.34	0.26	0.49	0.73

* Abbreviations: 3RG5G = 3-rhamnoglucosido-5-glucoside; 3G5G = 3,5-diglucoside; 3GG5G = 3-diglucosido-5-glucoside.

conditions. The identification of the acyl component, which is most frequently *p*-hydroxycinnamic acid or a similar organic acid, can also most conveniently be carried out by chromatographic means. The procedures for doing this have been already described[50–53]. The detection of such a compound, after alkaline hydrolysis of the pigment, confirms the identification of that pigment as an acylated anthocyanin.

The R_F values and structures of some typical acylated anthocyanins are given in Table 6. The fact that they are fairly clearly distinguishable in chromatographic behaviour from simple glycosides is illustrated by a comparison of R_F values of simple and acylated derivatives of pelargonidin (see Tables 2 and 6). As has already been mentioned, acylated derivatives have high R_F values in butanolic solvents, but relatively low R_F values in aqueous solvents. The R_F values of the acylated anthocyanins, shown in Table 6 are consistent with differences in structure and the expected regular relationships hold good. It is also to be noted that changes in the acyl group are sufficient to alter R_F values in one or more solvent systems.

One final point about the chromatography of acylated anthocyanins must be made. This is that, in general, they do not separate as satisfactorily as simple glycosides when present together in plant extracts. In some cases when two pigments such as the acylated 3-rhamnoglucosido-5-glucosides of pelargonidin and peonidin give

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clearly separated bands on chromatograms, it has been noticed that one of these bands is contaminated by the presence of the other pigment and one or more steps in purification are necessary to free the one pigment from the presence of the second pigment. Mixtures of acylated anthocyanins based on delphinidin, petunidin and malvidin are even more reluctant to separate on paper chromatograms.

7. EXAMPLES OF THE USE OF CHROMATOGRAPHIC METHODS

Chromatographic methods have largely replaced the ROBINSON colour and distribution tests for studying the widespread occurrence of anthocyanins in plants. Thus recent surveys of the anthocyanins in some tropical plants by FORSYTH AND SIMMONDS[27], in mountain plants by HAYASHI AND ABE[54] and in garden plants by REZNIK[10] have all been made by means of paper chromatography. The results are in general agreement with the earlier survey of LAWRENCE, PRICE, ROBINSON AND ROBINSON[12], in which the older methods were used. Chromatographic methods more clearly distinguish mixtures of pigments and the recent work has shown that the complexity of pigmentation in some plants is greater than was earlier supposed. The accuracy of the new methods is however no greater than that of the distribution tests, if identification is based solely on the measurement of R_F values and colour of spots in crude plant extracts. As has been mentioned, the R_F values of anthocyanins in such extracts can be affected by the presence of other components. In addition, a single anthocyanin may give rise to several spots on a chromatogram, if acid or enzymic hydrolysis of acyl or sugar groups takes place during the preparation of the extract. For these reasons, the wide range of values obtained by FORSYTH AND SIMMONDS[27] for cyanidin glycosides present in the plants of Trinidad, does not necessarily indicate a similar range of variation in glycosidic or acylated forms.

There is no doubt, however, that anthocyanins do occur in a greater number of different glycosidic combinations than the earlier work of ROBINSON AND ROBINSON[13] disclosed. The detailed chromatographic examination of more anthocyanins, on the lines of our recent investigation of pelargonidin derivatives[17], will surely reveal some further glycosidic variants.

Chromatographic procedures have also been used for studying in some detail the distribution of anthocyanins within a particular genus or order in connection with taxonomic investigations. A chromatographic examination of the anthocyanins of the petal, base and stamen of five species of *Papaver* has shown that the different species can be distinguished in this way[24]. This is because the distribution of the six anthocyanins in the flowering organs of *Papaver* plants is relatively complex. Chromatographic surveys of the anthocyanidins of the *Primulaceae*[14] and *Solanaceae*[55] have also yielded some results of taxonomic interest. At the same time, a new anthocyanidin, rosinidin was discovered[14] and on the basis of the chromatographic and spectral properties, a structure has been advanced for it. The identification of anthocyanidins formed from leuco-anthocyanins present in plant tissue has been made with the aid of chromatographic methods[40, 56].

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The most valuable application of chromatographic methods in the identification of anthocyanins has undoubtedly been and will continue to be in the field of biochemical genetics. The anthocyanins were the first group of plant substances in which the relationship between single genes and simple biochemical differences was demonstrated[57]. Although much work was done in this field by LAWRENCE[57], SCOTT-MONCRIEFF[58] and others, much remains to be done in more detailed and thorough investigations of the pigments present in the colour mutants of garden plants. The limiting factors in this type of investigation are shortage of plant material, the need for identifying all the pigments, including those present in trace amounts, and the difficulty of separating the complex mixtures encountered in the petals of hybrid plants. With the aid of paper chromatography, these difficulties can largely be overcome and considerable progress has been made recently in this field. Some of the results that have been obtained have been briefly summarised by the author[59]. A number of garden plants, in which flower colour variation occurs, have now been examined by chromatographic procedures. They include *Antirrhinum majus*[6], *Cyclamen europeaum*[60,61], *Dahlia variabilis*[7], *Impatiens balsamina*[62], *Primula sinensis*[63], *Streptocarpus*[64], *Solanum phureja*[15,49], and *Viola tricolor*[65].

Finally, chromatographic methods are useful for examining any anthocyanin of which the structure has not been fully determined by earlier investigators. There are many pigments which fall into this category. The presence in nature of new types of anthocyanidins is also revealed by chromatographic investigations[14]. Even if such pigments cannot be fully characterised by means of paper chromatography, it is at least possible to show that they are different from any of the known compounds. For example, the pigment of *Spirodela oligorrhiza*, thought to be a cyanidin glycoside by THIMANN AND EDMONDSON[66], has recently been re-investigated by GEISSMAN AND JURD[67] using chromatographic means. It is clear from these later results that the pigment is not directly related to any of the known anthocyanins.

From the above examples, it will be seen that chromatography has already established itself as a most valuable technique in the study of the anthocyanin plant pigments.

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PAPER CHROMATOGRAPHY OF INORGANIC PHOSPHORUS COMPOUNDS

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The methods used in the paper chromatography of inorganic and organic phosphates already show a certain standardization. At the same time these methods are being used to a great extent in solving special problems. This review tries to define the applications of the various methods:

A. General
B. Paper chromatography of inorganic phosphorus compounds
   1. Condensed phosphates,
   2. Phosphorus-oxygen compounds of lower valency states.

I. GENERAL INTRODUCTION

Any particular method of paper chromatography can be considered serviceable provided that a sharp separation of the substances is obtained, and if under constant conditions the R_F-values remain the same within a fixed limit of error. In general, certain conditions must be fulfilled to obtain a reproducibility of R_F-values[1,2].

(a) The temperature of the chromatography room should be constant to $\pm$ 0.5°, for the existence of a temperature gradient in the chromatographic chamber readily gives rise to irregular migration of the solvent front. As the distribution coefficient is a function of the temperature[3], the composition of the saturated phase is dependent on the temperature. Alterations in the composition of the solvent are accompanied by considerable variations in the R_F-values. When mixtures containing readily volatile components (ether, concentrated ammonia, ethyl formate, methanol) or readily esterified acid–alcohol mixtures are used, the working temperature should be less than 15°.

(b) For each chromatogram at least one reference compound must be used (guide chromatogram). If variations of greater than 0.02 from the usual R_F-value of a reference substance are obtained, the solvent mixture should be renewed. In the case of phosphorus compounds it is often necessary to allow greater variations (0.05)[2].

(c) The solvent mixture should be prepared and stored at the temperature of the chromatography room. It is better not to interpret the first run of a freshly prepared

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solvent, for the R_F-values are only reliable for the second run. In general, the mixture can be used for several days, but in routine investigations it should not be used more often than 7–8 times[4].

(d) If possible, the running times (heights of rise) should be kept constant. The composition and consistency of an ascending liquid layer cannot be assumed constant over the whole extent of the path, hence it follows that the R_F-value depends on the height of the front[5].

(e) No equilibration of the chromatographic paper is provided for in most of the methods considered here.

(f) In order to obtain comparable R_F-values, paper of the same production batch should be used.

In several recent investigations, attempts have been made to improve the sharpness of separation and the migration rate by cutting the paper in definite shapes[6,7].

Shape of paper

In the simplest and most widely used method, the paper sheets are cut into rectangular strips (Fig. 1, form 1). With this shape, a circular spot, or one with a favourable extension in the direction of flow, is obtained.

An approximate calculation based on standard conditions (1–2 mm³ of a 1% solution of phosphorus (as phosphate) gives a spot of 1.7–2.2 cm radius; if the solvent front ascends 25 cm from the starting point) shows that a minimum difference in

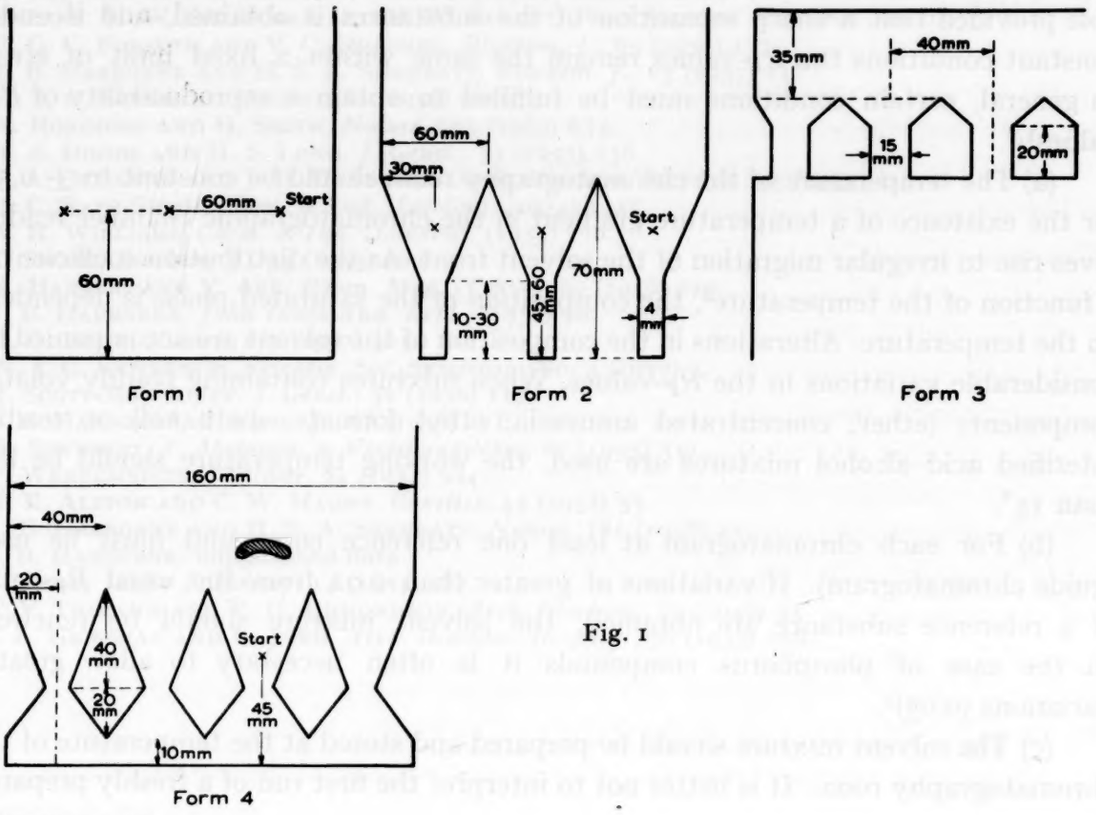

Fig. 1

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R_F-values of 0.055 is necessary if the spots are not to overlap. In practice, the separation of compounds with a difference in R_F-value of 0.06–0.09 is often very incomplete[7].

THILO AND GRUNZE[7] introduced tongue-shaped paper for the chromatography of phosphates (form 2). With this form, the main dimension of the spot lies parallel to the solvent front and perpendicular to the direction of flow. The sharpness of separation is considerably better than with form 1, but a longer running time is required.

For descending chromatography, the shape proposed by SCHWERDTFEGER[9] (form 3) permits a better separation while the running time is only slightly increased.

The writer has examined several new patterns from the view-points of sharpness of separation, amount of work and increased running times. With all the new forms, the process of solvent diffusion is still similar to that which occurs in circular paper chromatography. The solvent diffuses from a central point over a circle segment, encounters the test substance, and thus gives rise to the well-known dilution effect[10]. On the basis of these investigations, a new shape (form 4) was selected and experience has shown this to have advantages over previous forms. However, the optimum separations were still obtained by the circular paper method[11,40].

Sharpness of separation and zone shape. Interferences

When the new shape is used, substances with R_F-values of less than 0.6 appear in a characteristic kidney-shaped zone if interfering compounds are absent. With substances of high R_F-value, and especially substances which migrate in front, the zone degenerates to a straight line. The R_F-values and the shapes of the spots can be affected by displacement and boundary effects.

In our investigations on the chromatographic identification of reaction products with aliquot parts, a distinct displacement effect was observed. The spots were thus distributed in the sense of a maximum separation, *i.e.* they adopted a form and area which avoided overlapping. Typical displacement chromatograms can be formed by materials which undergo hydrolysis under the chromatographic conditions.

On the outer paths of the chromatogram next to the walls of the vessel, an uneven advance of the solvent front as well as a displacement, or shift, of the substance diffusing in the vicinity of the boundary towards that boundary can occasionally be observed. This effect is probably caused by temperature and decreases at lower working temperatures. For analytically important work, it is therefore recommended not to use the two outer paths[4].

In quantitative chromatography, interferences of this type are avoided by the following arrangement of the substances. One outer path remains empty, the other outer path is charged with the reference solution, and the inner path adjoining the first path is filled by the solution under investigation. Between the so-called reference strip which will be developed, and the strip which will be evaluated quantitatively, one path remains empty[4].

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Interferences in the paper chromatography of phosphorus compounds

A whole series of interfering effects becomes noticeable in the paper chromatography of phosphorus compounds:

(a) Overloading[12,13],
(b) Streaking,
(c) Lagging shadows[14],
(d) Tailing,
(e) Multiple zones,
(f) Inhibition and remaining at the starting point.

Ionic compounds should only be present up to certain definite limits which differ for individual compounds, otherwise an interference owing to the overloading effect occurs. Over the length of the chromatogram a continuous decrease in the water content in the direction of the flow can be assumed[15]. A high ionic concentration in any one position is accompanied by a higher water content and thus by an altered distribution; in these cases, the R_F-values become too high. The lower edge of the spot corresponds to a normal diffusion, whereas the centre and the upper limit may vary considerably[16]. When the water content decreases continually, it can readily be understood that difficultly soluble salts gradually precipitate and thus cause streaking.

Streaking can also occur when the solvent diffuses too quickly and distribution equilibrium is not established.

Another overloading effect occurs with unionized materials[12]. The diffusing solvent can transport the applied substance only up to a fixed concentration; when this is exceeded, the excess of substance remains behind and forms "shadows".

Interferences caused by ions. The above-mentioned interfering effects can also originate from the influence of polyvalent ions. The interference of foreign ions is normally demonstrated by a blurred, ill-defined spot which is generally deformed in some way (horse-shoe shaped, etc.). Probably cleavage of the spot can be traced to the same cause. Interfering cations occur (1) as trace impurities in the paper, (2) from the solvent used for the phosphorus compound, or (3) as the cation of the phosphate. Interfering ions on the paper can be removed by washing; an acid mixture is generally used and this must be followed by further washing until no more acid can be detected. To remove traces of heavy metals, it is advisable to place some hydrogen sulphide in the chromatographic chamber or to wash the paper with a complexing agent[14]. When Whatman paper is used, thorough washing with 8-hydroxyquinoline[14,17] or ethylenediaminetetraacetic acid[18] must be carried out. EGGLESTON AND HEMS[18] also add some ethylenediaminetetraacetic acid to the solvent.

Foreign ions associated with the phosphate solvent are a frequent phenomenon in the analysis of technical products (soaps, detergents) as well as in the preparation of biological materials for analysis. Interfering cations can be removed by a cation exchanger which converts the materials to their alkali metal salts. Where treatment with an ion exchanger does not seem advisable, a trace of a complexing agent,

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such as the sodium salt of ethylenediaminetetraacetic acid (EDTA) or pyridine ethylenedinitrilotetraacetic acid, can be added to the solution[19]. Of course, the R_F-values of esters are lowered by this treatment. GORDON *et al.*[12] recommend "over-spotting" the applied substance with a separate solution of pyridine sulphate: the sulphate ions fix the polyvalent cations and do not affect the diffusion of the anions.

Development of the chromatogram

After its removal from the solvent, the chromatogram is dried in air and then placed in an electric oven at 60–70° for some minutes[7].

1. *Standard method of* HANES AND ISHERWOOD[14]

The ammonium molybdate reagent of HANES AND ISHERWOOD has been applied as developer to all types of phosphorus compounds.

5 ml of 60% perchloric acid, 10 ml of 1 *N* hydrochloric acid and 25 ml of 4% ammonium molybdate solution are mixed and diluted to 100 ml with water.

After the spraying, the chromatogram is first dried in a current of warm air and then hung in an electric oven at 85° for 10 minutes.

The writer prefers to dry chromatograms of phosphoric acid esters and similar compounds at room temperature and then to heat at 70° for 7 minutes. Complete hydrolysis can be more readily guaranteed by this drying method than by rapid removal of water.

The disadvantage of this developer lies in its destructive effect on paper, which is due to the perchloric acid. The paper begins to decompose after a few days, but it can be stored in a relatively undamaged condition for several months when pasted on to paper in cases where photographic documentation is not possible. It is preferable to store the chromatogram in the dark in order to prevent after-blueing. SANSONI has studied the coloring effect of phosphates on filter paper and also investigated the efficiency of ammonium molybdate reagents of different concentration and composition[20]. He recommends a mixture of 1 vol. of aqueous 5% perchloric acid and 1 vol. of aqueous 1% ammonium molybdate solutions.

For chromatographic routine investigations an ammonium molybdate reagent containing nitric acid as the hydrolysing acid can also be used. PFRENGLE[4] prepared the reagent in the following way:

20 g of ammonium sulphate dissolved in 160 ml of water was mixed with 60 ml of concentrated nitric acid ($d = 1.4$). A solution of 60 g of ammonium molybdate in 160 ml of water was prepared at 50°, allowed to cool, and poured slowly with stirring into the nitric acid solution.

For details of photographic documentation, the paper by SANSONI should be consulted[20].

Development of the zones

The colour development of the phosphorus compound depends on the reduction of the phosphomolybdate complex formed, which then appears as a blue spot. Almost all common reducing agents can be used for this reduction.

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(*a*) *Photoreduction*[21]. SANSONI[20] has shown that the limit of sensitivity of the development with ultraviolet light or intense sunlight is the same (0.14 $\mu g/cm^2$ for $Na_2HPO_4 \cdot 2H_2O$) as with hydrogen sulphide. It is easier to obtain constant conditions and reproducible results when photochemical reduction is involved. The writer has found that the chromatogram can be readily developed by exposure to sunlight. It is often observed that the chromatogram becomes more distinct when exposed to light after chemical reduction, and this phenomenon depends on the same reaction. According to CROWTHER[13], it is sufficient to place the chromatogram under a strong UV-lamp for one minute.

(*b*) *Reduction with hydrogen sulphide.* After the chromatogram has been sprayed and dried, treatment with steam for a short time has been recommended[22]. This treatment is unnecessary for inorganic phosphates and readily hydrolysable organic phosphates.

Generally the chromatogram is left in the hydrogen sulphide atmosphere for 5–10 minutes. When the development is correct, the back-ground should remain white or slightly brownish, and the reduction must be controlled so that the chromatogram can be removed before the back-ground begins to turn blue. In qualitative chromatography, a favourable effect can be obtained by applying both methods.

(*c*) *Reduction with a sprayed reductant.* LEUTHARD AND TESTA[23] used a 0.1% solution of ascorbic acid as reductant in the chromatography of sugar phosphates (*cf.* GANGULI[24]). WESTMAN, SCOTT AND PEDLEY[25] have described tests with stannous chloride solution, and hydrazine hydrochloride has also been used as the reductant.

For industrial routine analyses, PFRENGLE[4] recommends a reducing mixture which is also suitable for quantitative colorimetric determinations, where it is predominantly applied.

28.8 g of $Na_2S_2O_5$ (anhydrous), 1.05 g of Na_2SO_3 (anhydrous) and 0.21 g of Photorex (monomethyl-*p*-aminophenolsulphate) are dissolved in water and diluted to 100 ml.

A possible disadvantage of this technique is that the spot can become blurred by frequent spraying.

2. *A variation of the ammonium molybdate method*

A variation of the above method was suggested by VELLUZ AND PESEZ[26]. The dried paper is sprayed with a nitric acid solution of ammonium molybdate containing a little basic quinine sulphate; the paper is then redried at 80° and examined under UV-light. Quinine phosphomolybdate appears as a dark spot against a blue fluorescent back-ground.

3. *Method of* WADE AND MORGAN[27]

(*a*) A fundamentally different method of revealing phosphates was adopted by WADE AND MORGAN. Iron(III) ions form a complex with phosphoric acid esters and

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when a solution of sulphosalicylic acid is sprayed on, the uncomplexed ferric ions form a light purple complex compound with sulphosalicylic acid.

The paper is sprayed with a 0.1% ferric chloride solution in 80% ethanol, dried at room temperature and again sprayed with a 1% sulphosalicylic acid solution in 80% ethanol. After drying, the phosphate appears as a white spot on a light purple back-ground.

The method is only suitable for use with acid solvents.

(*b*) The WADE–MORGAN method has been recommended in a slightly modified form quite recently by RUNECKLES AND KROTKOV[28].

The dried paper is immersed in a trough containing 1.5 g of $FeCl_3 \cdot 6H_2O$, 3 ml of 0.3 *N* hydrochloric acid and 970 ml of acetone, then redried and immersed in a solution of 12.5 g of sulphosalicylic acid in 1000 ml of acetone.

(*c*) Only one spraying is required in the method of MAIR–WALDBURG[11], who used a solution of ferric thiocyanate in butyl alcohol. (Two volumes of 0.1% ferric chloride solution, 1 volume of 1% ammonium thiocyanate solution and 3 volumes of distilled butanol are shaken and the butanol phase is used.)

(*d*) SHINAGAWA *et al.*[29] sprayed first with 0.01–0.005 *M* ferric chloride solution and then with 0.01–0.05 *M* ferrocyanide solution. Berlin blue is formed on the paper while the phosphate spots remain colorless or adopt a different colour.

The chromatography of ^{32}P-labelled phosphate

The radioactive labelled material is first separated by the usual methods of paper chromatography or electrochromatography[30]. The radioactive spots may then be determined on the paper by two methods:

(*a*) autoradiography,
(*b*) the scanning method.

Autoradiography

^{32}P emits a relatively energetic β-radiation. When a photographic plate is placed in direct contact with a "labelled" chromatogram, and then developed, darkened spots appear in positions opposite those occupied by ^{32}P or another "labelled" element on the paper. Autoradiography is predominantly a qualitative method, for the darkening of the emulsion is neither proportional to the amount of isotope nor linear with the radiated energy[31]. In the case of autoradiography with ^{32}P, only about 30% of the activity of the spots can be detected[32].

Scanning method

At the present time, the scanning method is of considerable importance, for quantitative determinations can readily be carried out. Autoradiography, which requires no complicated apparatus, has been applied very successfully in the investigation of intermediate metabolisms. Details are given by SANWAL[33] in *Papierchromatographie in der Botanik.*

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2. THE PAPER CHROMATOGRAPHY OF INORGANIC PHOSPHATES

A. Condensed phosphates

The paper chromatography of condensed phosphates was developed independently by EBEL in France, WESTMAN and his coworkers in Canada, and ANDO *et al.* in Japan. These investigators established the most suitable analytical methods as well as ion-exchange chromatography.

From the beginning, the method has provided convincing solutions to various analytical problems, *e.g.*:

(*a*) the examination of condensed phosphates described in the literature to establish their homogeneity and their classification,

(*b*) the identification of reaction products,

(*c*) the analysis of technical products (water softeners, detergents, food additives, tanning agents).

The method is reviewed by THILO AND GRUNZE in their monograph *Die Papierchromatographie der kondensierten Phosphate*[7]. The procedures described by THILO AND GRUNZE[7] can at present be regarded as standard methods, provided that the continual refinements and modifications are taken into account.

Paper

The papers used and their preliminary treatment are summarized in Table 1. A definite tendency exists at present to change over to harder varieties of paper, *e.g.* Schleicher and Schüll 2045b[38]. To prevent shrinkage of the paper, a final wash with 95% alcohol is to be recommended[28].

TABLE 1

Paper	*Reference*	*Preliminary treatment*
Schleicher & Schüll 2040a	THILO AND GRUNZE[7]	Washed with dilute hydrochloric acid and distilled water by the manufacturer.
Schleicher & Schüll 589	CROWTHER[13]	Untreated. S. & S. 589 black ribbon gives rapid separation of phosphates but a risk of overloading. S. & S. 589 orange ribbon gives slower separation but fewer interferences.
Whatman No. 1	WESTMAN *et al.*[25]	Wash for several days with acetic acid and then wash with distilled water to remove acetate completely.
Whatman No. 1 Whatman No. 4	EBEL *et al.*[34–37]	Wash with alcoholic 8-hydroxyquinoline solution, then with aqueous alcohol, 2 *N* acetic acid and distilled water.

Procedure for reference mixtures and compounds

The substances are applied on the starting point on a line which is situated at a distance of 3.5–5 cm from the immersed end of the paper. The starting line is placed high and is always situated within the cut-out part of the chromatogram; the R_F-values are somewhat smaller but the separations are cleaner[7].

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Sensitivity of detection

The lower limit of sensitivity probably lies at the value of 0.14 μg/cm^2 given by SANSONI[20] for disodium hydrogen phosphate dihydrate. In practice, the limit of sensitivity is said[4,34-37] to lie between 0.4 and 2 μg of phosphorus in a single spot, which corresponds to 0.15–0.66 μg of phosphorus per cm^2 of the spot size.

Reference solutions and substances[2,7]

Reference solutions should be prepared exclusively from sodium salts. Moreover, wherever possible, the substance under investigation should be applied as the sodium salt. Insoluble calcium phosphates are brought into solution by treatment with EDTA[2].

The reference solutions as well as the test solutions are usually prepared to contain 0.1% (to 1%) of phosphorus.

For example, a solution of 0.7 g of $Na_4P_2O_7 \cdot 10H_2O$ in 100 ml of water is used as a pyrophosphate standard; 0.001–0.01 ml of this solution, corresponding to 1–10 μg of phosphorus, is applied to the paper with a micropipette[10].

According to KARL-KROUPA[2], the capacity of the chromatography paper (Schleicher & Schüll 589) for short running times corresponds to a total content of 10 μg of phosphorus per applied spot. In addition, the amount of the phosphate mixture to be investigated should be kept small, so that small, well separated spots will be obtained on the chromatogram.

When several components are present in the mixture, it is possible to use a solution which contains 10–20 mg of phosphate mixture per ml of water; 0.001–0.01 ml of this solution is applied.

Analytical limits of the method

The group of condensed phosphates embraces compounds of condensation number $n = 1$ (orthophosphate) to condensation number 10^6 (Kurrol's potassium salt). It includes cyclic metaphosphates (tri- and tetrametaphosphate), chain oligophosphates (condensation number 4–10) and polyphosphates. The analytical problems involved can only be solved by the use of group solvents which separate the phosphates as individuals, or as groups with an average condensation number, in a definite R_F-range.

Experience has shown that the R_F-values are not always exactly reproducible even when the possible sources of error enumerated in section 1 are carefully avoided[2,4]. MORTIMER[39] has proposed that the distance travelled by a specific compound (orthophosphate) from the applied spot be chosen as the reference length rather than the distance travelled by the solvent front which is normally used. This so-called P_K-value (position constant) is defined as follows:

$$P_K = \frac{\text{Movement of the substance}}{\text{Movement of orthophosphate}} \times 100$$

THILO's *equation*[7]. If the P_K-values of particular condensed phosphates (the relationship is only valid for linear phosphates) are plotted against their condensation numbers

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n (the number of phosphorus atoms in the molecule), it can be seen that the P_K-values decrease exponentially with increasing condensation number. Thus the logarithms of the P_K-values plotted against the number of phosphorus atoms in the molecule lie on a straight line which complies with the formula:

$$\log P_K = -an + b \text{ (THILO's equation)}$$

where n is the condensation number and a and b are characteristic solvent constants.

With altered constants, THILO's equation is of course also valid for R_F-values.

The exponential decrease of the P_K-values corresponds to the behaviour of

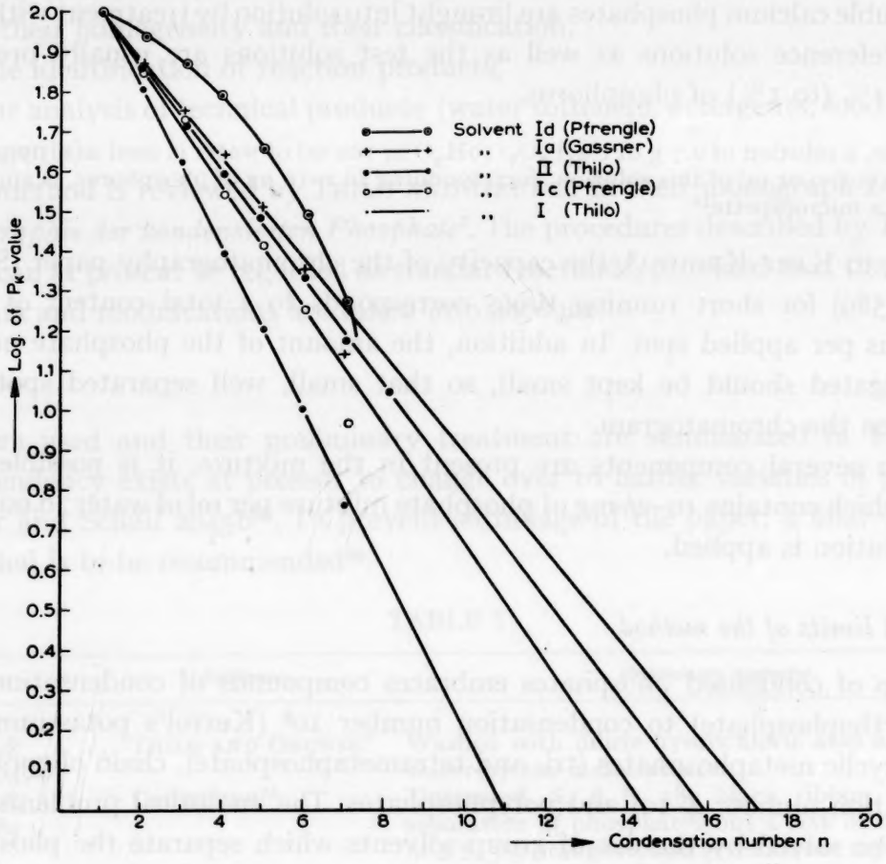

Fig. 2. THILO's equation for some solvents (see Table 2).

condensed phosphates on the chromatogram; for the higher condensed phosphates are placed close together in the vicinity of the starting point. THILO[7] has thus been able to define a practical limit to the migration. The straight line obtained from THILO's equation is extrapolated to a value $\log P_K = 0$, *i.e.* the intersect with the abscissa, and this indicates the condensation number. In this position, the P_K-value = 1, so that the compound of this condensation number travels exactly a hundredth part of the distance covered by orthophosphate and migrates practically no further.

Not all the solvents specified for condensed phosphates obey THILO's equation (see Fig. 2).

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TABLE 2

P_K VALUES OF CONDENSED PHOSPHATES

	S. I (THILO)		*S. II* (THILO)		*S. Ia* (GASSNER)		*S. Ic* (PFRENGLE)		*S. Id* (PFRENGLE)	
	P_K-*value*	*log*	P_K-*value*	*log*	P_K-*value*	*log*	P_K-*value*	*log*	P_K-*value*	*log*
Monophosphate $(PO_4)^{-3}$	100	2.000	100	2.000	100	2.000	100	2.000	100	2.000
Diphosphate $(P_2O_7)^{-4}$	63.8	1.805	72.6	1.861	73.7	1.867	71	1.851	86	1.934
Triphosphate $(P_3O_{10})^{-5}$	42.0	1.623	53.4	1.727	56.2	1.750	54	1.732	74	1.869
Tetraphosphate $(P_4O_{13})^{-6}$	24.6	1.391	39.7	1.599	43.7	1.640	35	1.544	60	1.778
Pentaphosphate $(P_5O_{16})^{-7}$	15.9	1.201	30.1	1.478	32.5	1.512	26	1.413	45	1.653
Hexaphosphate $(P_6O_{19})^{-8}$	10.1	1.004	21.9	1.340	22.5	1.352	18	1.255	31	1.491
Heptaphosphate $(P_7O_{22})^{-9}$	5.8	0.763	15.1	1.179	13.7	1.138	12.5	1.096	19	1.278
Octaphosphate $(P_8O_{25})^{-10}$			11.0	1.041						

TABLE 3

R_F VALUES OF CONDENSED PHOSPHATES

	S I R_F	*S II* R_F	*S Ia* R_F	*S Ib* R_F	*S Ic* R_F	*S Id* R_F	*S III* R_F	*S IV* R_F
Monophosphate $(PO_4)^{-3}$	0.69	0.73	0.80	0.79	0.70	0.79	0.33	0.41
Diphosphate $(P_2O_7)^{-4}$	0.44	0.53	0.59	0.62	0.50	0.68	0.24	0.31
Triphosphate $(P_3O_{10})^{-5}$	0.29	0.39	0.45	0.53	0.38	0.58	0.24	0.31
Tetraphosphate $(P_4O_{13})^{-6}$	0.17	0.29	0.35	0.49	0.25	0.47	(0.20)	(0.26)
Pentaphosphate $(P_5O_{16})^{-7}$	0.11	0.22	0.26	0.44	0.18	0.36	—	—
Hexaphosphate $(P_6O_{19})^{-8}$	0.07	0.16	0.18	0.32	0.13	0.25		
Heptaphosphate $(P_7O_{22})^{-9}$	0.04	0.11	0.11	—	0.09	0.15		
Octaphosphate $(P_8O_{25})^{-10}$	—	0.08	—	—	—	—		
Trimetaphosphate $(P_3O_9)^{-3}$	0.20	0.32			0.21	0.39	0.53	0.64
Tetrametaphosphate $(P_4O_{12})^{-4}$	0.08	0.18			0.13	0.22	0.40	0.50

S I = Solvent of EBEL[37], R_F-values of THILO[7]: 75 ml isopropanol, 25 ml water, 5 g trichloroacetic acid, 0.3 ml 20% ammonia.

S II = Solvent of THILO[7]: 70 ml isopropanol, 10 ml water, 20 ml 20% trichloroacetic acid, 0.3 ml 25% ammonia.

S Ia = Solvent of GASSNER[40]: 80 ml isopropanol, 5 g trichloroacetic acid, 0.3 ml 25% ammonia, 40 ml water, 40 ml ethyleneglycolmonomethylether.

S Ib = Solvent of GASSNER[40]: 80 ml isopropanol, 5 g trichloroacetic acid, 0.3 ml 25% ammonia, 70 ml water, 20 ml ethyleneglycolmonomethylether, 40 ml dioxane.

S Ic = Solvent of PFRENGLE[4]: 26.25 ml isopropanol, 13.75 ml water, 15.6 g trichloroacetic acid solution (containing 20 g trichloroacetic acid, 5.5 ml ammonia (d = 0.918) diluted to 100 ml with distilled water), 3 ml acetic acid solution (containing 20 ml 96% acetic acid and 80 ml water), 30 ml dioxane.

S Id = Solvent of PFRENGLE[4]: 60 ml methanol, 10.3 ml trichloroacetic acid solution (100 g trichloroacetic acid diluted to 500 ml and mixed with 22.7 ml ammonia (d = 0.918)), 5 ml acetic acid solution (20 ml 96% acetic acid and 80 ml water).

S III = Solvent of EBEL[34], R_F-values of THILO[7]: 40 ml isopropanol, 20 ml isobutanol, 39 ml water, 1 ml 25% ammonia.

S IV = Solvent of EBEL[37], R_F-values of THILO[7]: 30 ml isobutanol, 30 ml absolute ethanol, 39 ml water, 1 ml 25% ammonia.

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Standard mixtures according to THILO[7]

Acid solvent I (EBEL[37]); pH = 1.5, constant $a = 0.20$. Standard mixture for the separation of lower molecular poly- and metaphosphates up to tetramers.

75 ml of isopropanol, 25 ml of water, 5 g of trichloroacetic acid, 0.3 ml of 20% ammonia.

Conditions: ascending, temperature 16°, 14 hours.

Performance: THILO has established that migration ceases at a condensation number of approximately 11 P-atoms. The first six members of the condensed phosphates occupy 90% of the space traversed by the monophosphate ($P_K = 10$–100), and 9% of the remaining space is occupied by the phosphates P_7–P_{11}. All the higher condensed phosphates are found in the remaining percentage of the space. In this case, a difference in P_K-value of 4.8 corresponds exactly to a spot difference of 1 cm. The 5 polyphosphates in the P_K-range 1–10 cannot be more definitely separated.

Acid solvent II (THILO[7]); pH = 1.7, constant $a = 0.136$. Standard mixture for the simultaneous separation of phosphates of lower molecular weight and higher condensed oligomers.

70 ml of isopropanol, 10 ml of water, 20 ml of 20% trichloroacetic acid, 0.3 ml of 25% ammonia.

Conditions: ascending, temperature 16°, 14 hours.

Performance: At a condensation number of $n = 16$, no diffusion occurs. The R_F-values are distributed more evenly than with mixture I. The first eight members of the series in the P_K-range 10–100 are readily separated, whereas the polyphosphates P_8–P_{16} lying in the P_K-range 1–10 cannot easily be distinguished. Cyclic metaphosphates can only be detected with solvents I and II in the absence of higher condensed phosphates.

Acid solvent Ia (GASSNER[40]); pH = 1.7. This solvent corresponds to mixture I.

80 ml of isopropanol, 40 ml of water, 5 g of trichloroacetic acid, 0.3 ml of 25% ammonia, 40 ml of ethyleneglycolmonomethylether.

Conditions: ascending, temperature 32°, 30 hours.

Performance: By extrapolation, the condensation number at which migration no longer takes place is 17. The highest separation possible at present is obtained with the solvent of GASSNER[40]. In a polyphosphate glass with a total P_2O_5 content of 62%, corresponding to an approximate average condensation number P_5, 12 zones, *i.e.* P_{11} and P_{12}, can be differentiated on a circular paper chromatogram.

Acid solvent Ib (GASSNER[40]); pH = 1.7. This "dioxane mixture" is used for the most extensive possible separation of oligo- and polyphosphate groups.

80 ml of isopropanol, 70 ml of water, 5 g of trichloroacetic acid, 0.3 ml of 25% ammonia, 20 ml of ethyleneglycolmonomethylether, 40 ml of dioxane.

Conditions: ascending, temperature 12–14°, 18–20 hours.

Performance: THILO's equation cannot be strictly applied to this solvent. GASSNER[40] indicates that the limiting condensation number for migration is $n = 20$. The oligophosphates do not migrate as individuals; the oligophosphates and polyphosphates beyond the condensation number $n = 10$ are distributed very evenly over a distance of 20–30 cm.

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Acid solvent Ic (PFRENGLE[4]); this "dioxane mixture" corresponds approximately to solvent I.

26.25 ml of isopropanol, 13.75 ml of water, 15.6 g of trichloroacetic acid solution (containing 20 g of trichloroacetic acid and 5.5 ml of ammonia solution ($d = 0.918$) diluted to 100 ml with water), 3 ml of acetic acid solution (containing 20 ml of 96% acetic acid and 80 ml of water), 30 ml of dioxane.

Conditions: ascending, temperature 18–20°, 16–17 hours.

Performance: By extrapolation, the condensation number at which the migration ceases is 14. The rates of migration of monophosphate in solvent I of THILO and in solvent Ic of PFRENGLE are in an approximate ratio of 3:5. However, since the P_K-values are similar, this implies a favourable distribution of the phosphates over a greater distance.

Acid solvent Id (PFRENGLE[4]); "methanol mixture". This is a very fast flowing mixture suitable for qualitative orientation.

60 ml of methanol, 10.3 ml of trichloroacetic acid solution (100 g of trichloroacetic acid diluted to 500 ml to which 22.7 ml of ammonia ($d = 0.918$) are added), 5 ml of acetic acid solution (20 ml of 96% acetic acid and 80 ml of water)

Conditions: ascending, temperature 20°, 4 hours.

Performance: THILO's equation cannot be applied to the mixture. The P_K-values are distributed nearly equidistantly over the whole space. It is possible to separate metaphosphates from oligophosphates. The lagging shadows which appear are not noticeably detrimental to a qualitative evaluation. The danger of hydrolysis of the condensed phosphates is lessened when a solvent with such a short running time is used.

Ammoniacal standard solvents (THILO[7]). With ammoniacal solvents, trimeta- and tetrametaphosphates can be detected separately in presence of polyphosphates.

Ammoniacal solvent III (EBEL[34]).

40 ml of isopropanol, 20 ml of isobutanol, 39 ml of water, 1 ml of 25% ammonia.

Conditions: ascending, temperature 16°, 16 hours.

Performance: Tri- and tetrametaphosphates migrate fastest (P_K-values 160 and 125), and the separation of ortho- and polymeric phosphates is good. Di-, tri- and tetraphosphates cannot be sufficiently differentiated for identification purposes. REMY[41] used this solvent for the successful chromatographic detection of the phosphorophosphate $Na_3HP_2O_6$ ($R_F = 0.49$, $P_K \sim 110$).

Ammoniacal solvent IV (EBEL[37]).

30 ml of isobutanol, 30 ml of absolute alcohol, 39 ml of water, 1 ml of 25% ammonia.

Conditions: ascending, temperature 16°, 16 hours.

CROWTHER[13] used a mixture of *n*-propanol, ammonia and water (60:20:20 v/v).

Conditions: descending, room temperature, 48 hours.

Performance: With this mixture, a separation should be obtained up to condensation number $n = 9$. WESTMAN[42] used it successfully for the chromatographic detection

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of tetraphosphate. Apart from WESTMAN *et al.*[43] some other authors have favoured the descending method, for it permits the whole length of the sheet to be utilized for the separation of compounds[44].

Two-dimensional methods

KARL-KROUPA[2] has elaborated the two-dimensional technique of EBEL[36] to allow a clean separation of cyclic metaphosphates and condensed phosphates. First, the faster flowing metaphosphates are separated from the other phosphates with the ammoniacal solvent III; then, in the unused part of the paper, a reference solution is applied on a second starting line which is perpendicular to the first, and migration is started in the new direction with an acid solvent. After the first run, the paper must be dried only at room temperature, in order to avoid hydrolysis as much as possible.

Using this method in investigations on the hydrolysis of GRAHAM's salt, VAN WAZER and KARL-KROUPA[45] obtained several distinct spots which must be explained as the higher metaphosphates (penta- and hexametaphosphate) which had long been sought.

Detection of spots

Various attempts have been made to detect specific compounds selectively. Orthophosphate appears immediately as the definite yellow colour of phosphomolybdate on development with the ammonium molybdate reagent independently of the solvent used. After reduction (with any reductant) it gives a gray-green colour which differentiates it from other phosphates[7]. According to REMY[41], phosphorophosphate also shows this gray colour on the chromatogram. After some time, a yellow colour may also occur with higher condensed phosphates.

The yellow phosphomolybdate complex gives a definite fluorescence in ultraviolet light and the intensity maximum and geometrical boundaries of the fluorescence coincide with those of the yellow colour observed in visible light. The molybdenum blue spots finally produced by reduction are often displaced and accordingly have their intensity maxima in different places[13].

SANSONI[20] has observed that in many cases only the edges of the spots are coloured while the interior remains white. The writer has observed the same effect in typical displacement chromatograms. This interference probably depends on the overloading of the positions, possibly with quite different substances which are not revealed on the chromatogram.

In an early work, ANDO, ITO, ISHII AND SODA[46] developed in several steps: (1) spraying with a 4% solution of ammonium molybdate in 8% nitric acid and heating, (2) spraying with a 0.05% solution of benzidine in 10% acetic acid, (3) treatment with ammonia vapour. By this treatment, pyrophosphate gave a purple red spot.

Special conditions for the chromatography of condensed phosphates. Interferences and their removal

(*a*) *Hydrolysis.* Polyphosphates remain practically unaltered in strongly acidic solutions for any length of time[47].

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The question of how great are the secondary changes which can be expected with condensed poly- and metaphosphates under the conditions of chromatography, is particularly important when quantitative methods are considered. Quantitative investigations of pyro- and triphosphates have shown that the rate of hydrolysis should be the same as that which would occur in aqueous solutions of corresponding acidity or basicity. It is therefore possible to introduce in quantitative paper chromatography correction factors for the hydrolysis; these are obtained from corresponding measurements in aqueous solution[2]. The hydrolysis becomes more important, the longer the running time and the higher the working temperature during the process. An extraordinary increase in hydrolysis can be observed under the catalytic influence of impurities, micro-organisms and polyvalent cations[2]. Hydrolytic cleavages and changes in the condensed phosphates can occur under the polarizing influence of the field of polyvalent cations[48].

(*b*) *Interferences from foreign ions.* GASSNER[40] has made a thorough investigation of the effect of foreign ions in various concentrations on the paper chromatography of condensed phosphates. It has been shown that even small amounts of nearly all heavy metals interfere with the chromatographic separation. Probably, different reactions which are due to the heavy metal ions (complex formation, acceleration of hydrolysis) are superimposed on each other. In earlier investigations, complex formation of oligo- and polyphosphates with metals, even with the alkali metals, has been detected[49].

Today, the phenomena which were previously ascribed to the formation of complexes of polyphosphates ($n > 3$) with cations, are mainly explained as ion-exchange processes[47]. In the case of complexes of the alkaline earth metals with ATP, fructosephosphate and glycerophosphate, the phosphate radicle is the complex-forming part of the molecule[50]; with such complexes, the transport mechanism is likely to be different from that of the unchelated ions. The cyclic metaphosphates, which cannot form chelates[51], form much less stable complexes than the linear phosphates. This conception is strengthened by observations of GASSNER[40] who noted that trimetaphosphate migrated while the other phosphates remained at the start in presence of Mg^{+2}, Sr^{+2}, Ba^{+2}, Zn^{+2}. With silver trimetaphosphate, the writer has observed a brown halo of silver sulphide at a distance of about 7 cm from the start after reduction with hydrogen sulphide, whereas the trimetaphosphate spot appeared at a distance of 4.2 cm, corresponding to a R_F-value of 0.18 under normal conditions[7].

In investigations of chromatographic separation processes, LEDERER[52] established that the migration of orthophosphate in butanol-hydrochloric acid medium is not detectably affected by the presence of ferric, cupric or uranyl ions in concentrations of 10 mg/ml. At the end of the process, the orthophosphate is situated some centimetres in front of the iron. However, radiactive labelled $H_3{}^{32}PO_4$, in tracer amounts, is somewhat retarded by ferric ions in the chromatographic separation.

Whenever polyvalent cations are present in the solution, a certain amount of the phosphate generally remains at the start[40]; pyro- and trimetaphosphate have been specially investigated. THILO[53] states that calcium trimetaphosphate and tetrameta-

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phosphate also remain at the start in the same way as a highly polymerized phosphate. THILO has proved by precipitation of the calcium ions with oxalate and renewed chromatography that no secondary change of the chromatographed trimetaphosphate occurs[53]. Probably the cations act as exchange cations between the cellulose system, with its statistically distributed negative charge[54], and the phosphate radicles in solution, and thus facilitate the linkage with the paper.

According to GASSNER[40], the interference can be removed wholly, or at least partly, by the addition of Complexone III (disodium salt of ethylenediaminetetraacetic acid). The detrimental effect of calcium up to 16 μg per applied spot can be eliminated by Complexone addition and alkaline chromatography. The optimum conditions for chromatography are obtained when the substances are converted to the sodium salts of the acids concerned by means of a cation exchanger in the sodium form[53]. KARL-KROUPA[2] found that treatment with ion-exchangers does not remove calcium ions completely from the solution.

Interfering anions. High salt concentrations generally interfere noticeably[40]. Sulphate, sulphite and formate affect the separations in alkaline solvents while chromate interferes particularly with acid chromatograms.

Arsenate causes a blue or brown spot in the orthophosphate position, but with predominantly organic solvents, silicate remains at the start as the blue reduced silicomolybdate complex[55].

Quantitative paper chromatography of the condensed phosphates

The quantitative determination of a single condensed phosphate necessitates a particularly sharp separation of the components of the mixture. Moreover, the spot must be cut out in such a way that the whole area of the spot is removed with certainty. Therefore it is often preferable to separate groups of a definite range of condensation number and to determine the total content of the group, rather than to carry out single determinations of each condensed phosphate. Fortunately, it is not necessary to determine the absolute amounts of the separate phosphates; provided that the proportion of the phosphate content of each spot is obtained, the absolute percentage can be calculated from a total phosphorus determination which is carried out simultaneously. Thus, errors arising from the application of the solution are not carried over to the determination procedure, for only the proportions of the various components are determined.

The choice of method depends on the purpose of the analysis (see *Die Papierchromatographie der kondensierten Phosphate*[7]). First, a qualitative chromatogram should be made in order to obtain information on the composition of the sample.

For the determination of small amounts of orthophosphate in presence of condensed phosphates, GASSNER[40] did not carry out a chromatographic separation but simply measured photometrically the molybdenum blue produced by treatment with ammonium molybdate and ascorbic acid at pH = 4. Direct evaluation of the phosphate spots by size comparison or photometric measurement is not practicable, because the blue complex formed on the paper is not homogeneous[2]. Thus the quan-

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titative determination of phosphorus compounds involves several essential steps:

(a) separation of the phosphorus-containing substances on paper,
(b) hydrolysis to orthophosphate,
(c) conversion of orthophosphate to molybdenum blue by a reducing solution,
(d) colorimetry.

There are two distinct methods of proceeding.

1. A reference strip identical with the chromatogram is developed, and the corresponding spots on the chromatogram are cut out "blindly" and determined quantitatively according to the methods given on p. 242[56]. The division of the paper can be made according to the directions on p. 227. Solvents Ic and Ib (pp. 236, 237) are extremely suitable for this "blind" method because of the even distribution of the phosphates over the distance of the solvent flow.

KÖBERLEIN AND MAIR-WALDBURG[11], who used circular paper chromatography, identified the separate phosphate rings by spraying with a solution of ferric thiocyanate in butyl alcohol (p. 231). Since filter paper does not interfere with the hydrolysis[2], PFRENGLE[4] carried out the elution and the conversion to orthophosphate in a single step.

The divided zones of paper were treated with 50 ml of 1 *N* sulphuric acid overnight at 90° in a 100 ml Erlenmeyer flask.

Phosphates of high molecular weight cannot be completely detected by this procedure and are boiled for 2 hours under a reflux condenser: complete hydrolysis and dissolution from the paper is thus obtained. PFRENGLE used the colorimetric method of SCHEEL-LEDERLE[57], for the solutions are stable for a longer time and the reduction solution is suitable for the development of reference strips as well as for the colorimetric method.

In another procedure, the paper is first mineralized and then the hydrolysis of the phosphates is carried out[11].

2. The phosphorus compound is first made visible on the paper by means of the molybdenum blue method. After the development, the phosphate spots are cut out and the phosphate is brought into solution by *extraction* or by *ashing* of the paper. KING[58] preferred to ash the paper by heating with some drops of concentrated nitric acid for one hour at 150° on a sandbath. The final solution must be completely clear and must not contain nitrogen oxides, for even traces of these affect the colorimetric determination. Apparently, high colorimetric values are obtained when the phosphate compounds developed with the ammonium molybdate reagent are ashed[11].

Since the molybdenum complex is not readily soluble in acid solutions, it is preferable to extract with dilute ammonia[2] (usually 0.1 *N* ammonia), neutralize with dilute perchloric acid or 8 *N* sulphuric acid and then add excess of acid for the hydrolysis. THILO AND WIEKER[7] neutralized to *p*-nitrophenol indicator before the phosphomolybdate complex was formed, for the colour intensity of molybdenum blue is strongly dependent on the pH of the solution. The sodium hydroxide used for the neutralization should be stored in a polythene or silver vessel, because moderate

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amounts of silicic acid dissolved from glass cause errors in the colorimetric phosphorus determination.

The elution of the complexes with ammonia can be omitted provided that the colorimetric determination of each phosphate fraction resulting from an analysis is carried out under exactly the same conditions[2]. In the transference of the phosphomolybdate complex from the organic phase, there is of course established a distribution equilibrium between the aqueous phase, the cellulose system of the paper, and the organic phase. This causes a negative error in the final colorimetric determination of the extracted complex, but it can be eliminated by excising always exactly the same amount of paper and by extracting according to exactly the same procedure. The proportions of the separate phosphates obtained in this way can be converted to absolute percentages when the total phosphorus content of the sample is known. When the initial extraction with ammonia is omitted, the accuracy of the determination is obviously somewhat less for smaller amounts of phosphorus[2].

Extraction of the phosphomolybdate complex with organic solvents (isobutanol)

Extraction of the complex with isobutanol is advantageous because the determination of the phosphorus content by optical methods can be carried out without an appreciable "salt error", *i.e.* without the effect of a non-absorbing material on the light absorption[60]. Detailed procedures are given by WIEKER[7] and KARL-KROUPA[2].

For example, THILO AND WIEKER[7] recommend that the following solutions:

1. 0.06 ml of 10 *N* sulphuric acid,
2. 0.25 ml of 5% ammonium molybdate solution,
3. the neutralized test solution,
4. 5 ml of distilled isobutanol,

be placed in a separatory funnel and shaken for at least 20 seconds. After separation, the aqueous phase is washed with 1 ml of 1 *N* sulphuric acid.

Various reductants have been suggested for the reduction to molybdenum blue[61].

(*a*) Stannous chloride solution has been used by WIEKER[7], KARL-KROUPA[2], WESTMAN[25] and KÖBERLEIN[11].

The usual solution (WIEKER[7]) is prepared freshly each day from 0.25 ml of stock solution (10 g of stannous chloride and 25 ml of concentrated hydrochloric acid) and 25 ml of 1 *N* sulphuric acid.

(*b*) Reduction with Metol (Photorex, etc.) was suggested by PFRENGLE[4,57] (see p. 230).

(*c*) Reduction with hydrazine hydrochloride[13].

(*d*) Reduction with ascorbic acid[44].

It is only to be expected that the results of critical examinations of colorimetric methods for phosphorus[60,62] will also be applicable to the procedures of quantitative paper chromatography.

Sources of error in quantitative methods

Considerable errors can arise from working with micro-volumes and in the application of the sample solutions. Therefore several recent workers avoid the determination of absolute amounts; only the proportion of the total phosphate content contained in

each separate spot is estimated[2,40]. The absolute percentage can then be calculated from a determination of the total phosphorus content of the sample.

The amounts of phosphate to be determined, and hence the absolute accuracy of the measurements, are defined by the chromatographic procedures. For correct chromatographic separation, not more than 200 μg of phosphate (calculated as P_2O_5) in an average 10 μl of solution should be used. The permissible concentration of phosphorus in the colorimetric method depends on the particular colorimetric procedure used. When the test substance consists mainly of polyphosphate, which does not migrate, a slightly larger sample can be taken. For the determination of constituents of high molecular weight, a correspondingly smaller fraction of the solution should be submitted to colorimetry.

The usual sample for chromatographic analysis corresponds to about 100 μg of P_2O_5. If the absolute error of the photometric determination is established as 0.5 μg of P_2O_5, then the phosphate compounds can be determined with an absolute accuracy of $\pm$ 0.5%[2,13,40]. The relative error for components of a mixture which are present in smaller amounts, is correspondingly greater. WIEKER specifies a maximum relative error of 3%. The phosphorus content of the chromatographic paper can be neglected[7] provided that is does not exceed 0.01 μg P/cm^2.

Radioactive labelled condensed phosphates

Working details of the scanning method are given by MEISSNER[63]. The limit of sensitivity is of the order of 10^{-9} g. To prepare a distribution curve, the distribution of ^{32}P on the chromatogram is measured by means of a Geiger-Müller counter tube, and the measured activity is plotted as a function of the distance from the starting point (or of the R_F-value). The areas under the maxima of the distribution curve are proportional to the amounts of phosphorus situated on the corresponding parts of the chromatogram. The relative amounts of the separate phosphates can be established directly by analysis of the curve and plane measurements. For absolute determinations of phosphorus it is necessary to carry out absolute calibration of the measurement apparatus.

The radioactive method has proved very suitable in the investigation of intermediate metabolisms with phosphates[64].

B. Compounds containing oxygen and phosphorus in lower valency states

The lower oxygenated acids of phosphorus can be chromatographed by the same methods as the condensed phosphates. EBEL[37] first reported that hypophosphite, phosphite, pyrophosphite and orthophosphate cannot be separated in acid solvent I; however, it is possible to separate this group from hypophosphate or pyrophosphate. The separation of arsenite and arsenate proceeds smoothly. The separation is also unsatisfactory with the mixture suggested by D'AMORE (70 ml of isobutanol, 6 ml of acetylacetone, 2.5 g of trichloroacetic acid, 0.2 ml of ammonia (d = 0.91) and 39 ml of water)[65]. The alkaline mixtures offer better possibilities of separation. The five

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anions, hypophosphite, phosphite, pyrophosphite, hypophosphate and orthophosphate, were readily separated by EBEL, who used the ammoniacal solvent IV. Of course, the separation of arsenite and arsenate is not then possible. Ammoniacal solvent III has also been applied[65].

BONNIN AND SUE[66] carried out separations of phosphate, phosphite and hypophosphite with a mixture of *n*-butanol, dioxane and 1 *N* ammonia (1 : 1 : 1).

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SEPARATION OF ISOTOPES BY CHROMATOGRAPHY AND BY ELECTROPHORESIS

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1. INTRODUCTION

Since the discovery of isotopes by ASTON and the brilliant work of HERTZ on the separation of ^{20}Ne and ^{22}Ne, the separation of isotopes has been very widely investigated. With the development of the nuclear industry, the possibility of separating isotopes in considerable amounts by economically profitable methods has become a subject of wide-spread interest and many workers have found it necessary to pay particular attention to physico-chemical methods. Moreover, from the theoretical point of view, such studies are valuable for establishing the mechanism of physico-chemical reactions by measurement of isotopic effects.

In the past few years, chemistry has acquired two new techniques, chromatography and electrophoresis, by which it has been possible to achieve reputedly difficult separations such as those of the rare earths or the various amino acids. These two methods have also been applied to the separation of isotopes and this review covers the results which have been obtained in this field.

These techniques are most often used in a liquid medium, hence they are especially suitable for the separation of isotopes of those elements and metals which do not give gaseous compounds and to which techniques of diffusion, distillation and chemical exchange are inapplicable. Chromatography and electrophoresis appear to be the only practicable techniques for these elements. The most important element of this type is lithium, for the separated isotopes find many applications in atomic energy work. The isotope ^{6}Li gives rise with efficiency to the nuclear reaction $^{6}Li(n,\alpha)^{3}H$ which serves for the preparation of tritium, whereas ^{7}Li has a very low cross-section for neutron capture and can be used in the metallic state as a liquid heat exchanger in energy-producing reactors.

2. CHROMATOGRAPHY

The basic mechanisms, and thus the possibilities of separation, of the many processes of chromatography are quite different. For example, no work has been published to date on the use of partition chromatography for the separation of isotopes. Some separations have been accomplished by adsorption chromatography in the gaseous phase, but in the liquid phase only ion-exchange methods have proved successful.

Whichever chromatographic mechanism is involved, the separation is based on

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the equilibrium of partition of two or more isotopes between two phases. From this point of view, the chromatographic column is analogous in many ways to a fractional distillation column, and this is why the length of a column is often expressed as the number of "theoretical plates" which it represents. In isotopic separations, the number of plates should be as high as possible, for a complete separation of two isotopes is never obtained; the fractions are only more or less enriched in one or the other of the isotopes. Hence GLUECKAUF[1] has studied theoretically the influence on the number of plates of various factors, such as the grain size, the volume of interstitial liquid and the flow rate, in relation to the diffusion constants in the two phases. For a given column, an approximate knowledge of the number, p, of theoretical plates can be obtained provided that certain simplifying hypotheses are allowed. The principal of these are (a) the adsorption isotherm is linear, (b) the flow rate of liquid is slow enough for the two phases present to be constantly in equilibrium. If it is assumed that these conditions are fulfilled, the number of theoretical plates p can be calculated according to a theory developed by MAYER AND TOMPKINS[2], by studying the shape of the elution curve of a given element. In general, this number p varies with the element under study.

Each mechanism of chromatography is characterized by its elementary separation factor, α, which is equal to the quotient of the isotopic ratios of the element in the two phases in contact. If R_1 is the isotopic ratio in the liquid phase and R_2 is the isotopic ratio in the adsorbed phase, then $\alpha = R_1/R_2$. This ratio can be determined directly by carrying out an isotopic analysis of the two phases when equilibrium is attained, but the precision is poor because the difference of the isotopic abundances is extremely small. The factor α can be obtained more precisely by measuring the variation of the isotopic ratio along the elution band if the number of theoretical plates p is known, and by applying the theory of MAYER AND TOMPKINS which states that log R is a linear function of the eluted volume.

(a) Ion-exchange chromatography

The earliest work in this field is that of TAYLOR AND UREY[3]. These authors prepared an artificial zeolite of approximate composition, $Na_2O \cdot Al_2O_3 \cdot 6SiO_2 \cdot xH_2O$, in which the sodium can be replaced by other cations. When the zeolite in the sodium form is placed in contact with a salt solution containing the cation A^+, an ionic equilibrium is set up after a few minutes:

$$NaZ + A^+ \rightleftarrows AZ + Na^+$$

This equilibrium is governed approximately by the law of mass action and for two isotopes, the equilibrium constants are slightly different.

TAYLOR AND UREY made a special study of the separation of lithium isotopes and tried to determine the separation factor α directly by the method of phases in equilibrium:

$$\alpha = \frac{^6Li}{^7Li\ (\text{zeolite})} : \frac{^6Li}{^7Li\ (\text{solution})}$$

However, since the difference in behaviour of the two isotopes is slight, the extractions must be repeated many times. The method chosen consisted of extracting a

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large volume of lithium chloride solution with numerous successive small amounts of zeolite, so that the distillation formula of RAYLEIGH could be applied:

$$\left(\frac{1-N_0}{1-N}\right)^{\frac{1}{\alpha-1}} \left(\frac{N}{N_0}\right)^{\frac{\alpha}{\alpha-1}} = \frac{W_0}{W}$$

where N_0 is the fraction of heavy isotope in the initial amount W_0 of the material and N is the final fraction in the final amount W.

In practice, a solution containing 300 g of lithium chloride was extracted by many successive portions of 30 g of sodium zeolite. After the amount of lithium had been reduced by a factor of 70, the ratio $^7Li/^6Li$, which was initially 11.6, attained a final value of 12.7 so that the separation factor α was 1.022. The isotopic analyses were carried out by mass spectrometry in an apparatus similar to that described by BREWER[4].

More recently, GLUECKAUF[1] again studied lithium isotopes in order to verify quantitatively the equations deduced from his theory for the calculation of the height of theoretical plates. He used a column 90 cm long, filled with the ion-exchange resin zeo-karb H.I. and a solution of lithium acetate as solvent; the separation of the isotopes was studied by a method involving boundary migration. The isotopic analysis was made by applying the nuclear reaction:

$$^6_3Li + n \longrightarrow {}^4_2He + {}^3_1H + 4.6\ MeV$$

For the analyses, each sample was evaporated on a platinum disc and introduced into a small ionization chamber containing a Ra-Be neutron source; the number of impulses counted was then proportional to the amount of ^6Li present. In these experiments, GLUECKAUF obtained a head fraction in which the concentration of ^6Li was only 0.5% as compared with 7.5% in the natural sample. He did not interpret the result in detail but only concluded that the separation factor must be much lower than that found by TAYLOR AND UREY.

It is worth noting the work of GROSSE[5] who eluted a band of lithium on a column 145 cm in length filled with a sulphonated divinyl styrene resin. The head fraction, corresponding to 0.05% of the total lithium, contained 94.6% ^7Li whereas the tail fraction, corresponding to 0.2% of the total lithium, contained only 89.6% ^7Li. MÉNES, SAÏTO AND ROTH[6] have carried out more recent work on lithium. They used a 1 m column of Dowex-50 resin and studied the elution of a 0.4 cm zone of lithium, corresponding to 150 mg of lithium, by 0.3 N sulphuric acid. The elution curve (Fig. 1) agreed with that predicted by the theory of MAYER AND TOMPKINS, the number of theoretical plates being about 4,000. The variation of the isotopic ratio along the elution band allowed the separation factor α to be deduced and values between 1.001 and 1.002 were found. The isotopic analyses were done by mass spectrometry as well as by neutron irradiation.

A similar technique has been used by BETTS *et al.*[7] to study the separation of the two radioactive isotopes of sodium, ^{22}Na and ^{24}Na. The former emits β^+- and γ-rays with a period of 2.6 years whereas ^{24}Na emits β- and γ-rays with a period of 14.8 hours.

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These two isotopes are very suitable for the measurement of isotopic effects, for their relative difference in mass is high ($\Delta M/M = 9\%$), and the ratio $^{22}Na/^{24}Na$ can be measured accurately by following the decrease in radioactivity as a function of the

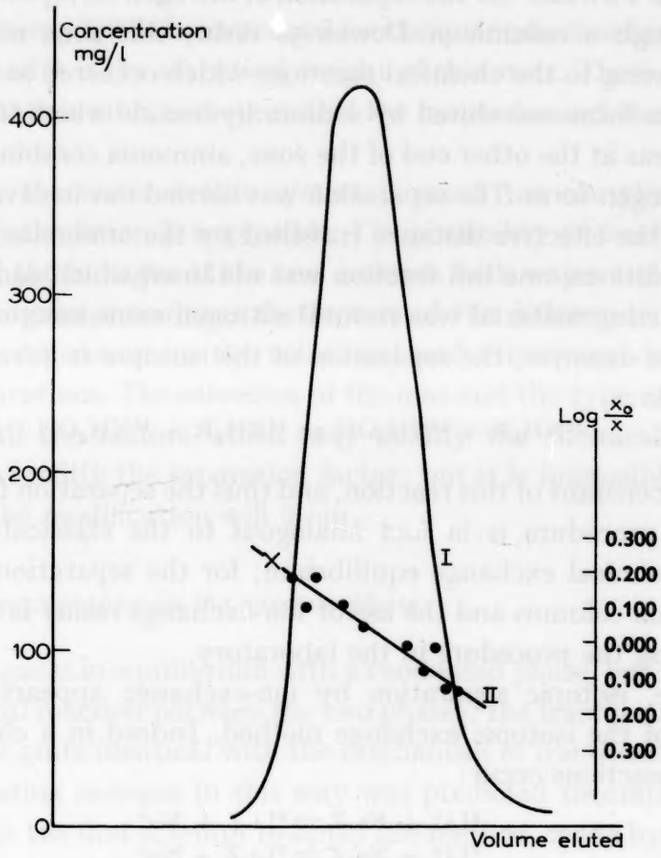

Fig. 1. I: Elution curve of LiCl on Dowex-50. II: Ratios $^7Li/^6Li$ expressed as log x_0/x. (According to MÉNES, SAÏTO AND ROTH[6]).

time. In principle, the isotopic ratio[13] is determined by two measurements of the activity a and a' at times t and t' about 48 hours apart:

$$a = \exp.(-\lambda t)^{24}Na + {}^{22}Na$$
$$a' = \exp.(-\lambda t)^{24}Na + {}^{22}Na$$

The resolution of these equations yields the values of ^{22}Na and ^{24}Na extrapolated to time $t = 0$ chosen as the origin.

In general, the use of radioactive isotopes is very advantageous, for it is then easy to measure rapidly a great number of isotopic ratios. In addition, it is possible to measure isotopic effects with elements which possess only one stable isotope such as sodium and cesium. In the work of BETTS *et al.*, where a zone of a mixture of ^{22}Na and ^{24}Na was eluted on a 1 m column of Dowex-50 resin, it was possible to observe variations in the isotopic ratio which amounted to a maximum of 10% from one end to the other of the eluted zone; the deduced separation factor was extremely low ($\alpha = 1.00016$). It is of interest to note that these authors observed a very considerable decrease in the isotopic separation when the temperature of the column was raised;

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however, the effect was attributed more to an increase in interferences than to a decrease in the separation factor.

The most remarkable results in ion-exchange chromatography are those obtained by SPEDDING AND POWELL[8] for the separation of nitrogen isotopes. A zone of ammonia was eluted through a column of Dowex-50 resin; this zone maintained its edges clearly defined owing to the chemical reactions which occurred at each end. The resin in the ammonium form was eluted by sodium hydroxide which liberated ammonium hydroxide, whereas at the other end of the zone, ammonia combined directly with the resin in the hydrogen form. The separation was carried out in several columns placed in series so that the effective distance travelled by the ammoniacal zone was 200 m. Under these conditions, one tail fraction was obtained which contained 74% of ^{15}N, although the starting material was natural nitrogen containing only 0.365% of ^{15}N.

In the above example, the separation of the isotopes is governed by a chemical exchange reaction:

$$^{15}NH_4R + {}^{14}NH_4OH \rightleftarrows {}^{14}NH_4R + {}^{15}NH_4OH$$

The equilibrium constant of this reaction, and thus the separation factor α, corresponds to 1.0257. This procedure is in fact analogous to the classical method of isotope separation by chemical exchange equilibrium; for the separation of ^{15}N can also be done in distillation columns and the use of ion-exchange resins is simply a convenient means of applying the procedure in the laboratory.

In principle, isotopic separation by ion-exchange appears generally to be a particular case of the isotopic exchange method. Indeed in a chromatography, two ionic exchange reactions occur:

$$^{6}Li^+ + Na.Z \rightleftarrows {}^{6}Li.Z + Na^+$$
$$^{7}Li^+ + Na.Z \rightleftarrows {}^{7}Li.Z + Na^+$$

and the separation of isotopes depends on the fact that the equilibrium constants of these two reactions are not identical. It can be deduced that the equilibrium constant of the reaction:

$$^{7}Li^+ + {}^{6}Li.Z \rightleftarrows {}^{6}Li^+ + {}^{7}Li.Z$$

is slightly different from 1.

In theory it is possible to predict the equilibrium constants of these isotopic exchanges by applying statistical thermodynamics; but in practice the calculations are only possible in the simple cases where the molecules in equilibrium are in the gaseous state. UREY AND GREIFF[9] were the first to develop a theory by which the equilibrium constants of many isotopic exchange reactions can be calculated starting from molecular spectra data. For any reversible reaction

$$aA + bB = \cdots \rightleftarrows mM + nN + \cdots$$

the mass-action constant $K = P_A^a \cdot P_B^b / P_M^m \cdot P_N^n$ is equal to the ratio of the partition functions f_A of the various molecules:

$$K = \frac{f_M^m \cdot f_N^n}{f_A^a \cdot f_B^b}.$$

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The partition function f of each molecule contains the various energy levels of rotation and vibration in the molecule:

$$f = \sum_{v,j} p \exp. - \frac{E(j,v)}{kT}$$

where $E(j,v)$ is an energy level characterized by the quantum numbers of rotation and vibration j and v, and p is the statistical weight of this state. These energy states are known from the rotation-vibration spectra of the molecules and thus the constant K can be calculated.

Unfortunately, the water–zeolite system is much too complicated and UREY[3] himself states that calculation is impossible. The possibility of fractionation depends on the difference of the linkages of the two isotopes with water and with the zeolite. The equilibrium constant introduces the factors of the differences of energy at the zero point and the differences in the rotational and vibrational frequencies of the two isotopic configurations. The solvation of the ions and the type of zeolite also play important parts. All the factors which may modify the chemical linkages or the solvation, may also modify the separation factor; but it is impossible to predict how or to what extent the modification will occur.

(b) *Adsorption chromatography in the gaseous phase*

In this method, the gas is in equilibrium with a condensed phase, but there is, properly speaking, no chemical reaction between the two phases; the fractionation process then can be considered as quite identical with the mechanism of fractional distillation. The possibility of separating isotopes in this way was predicted theoretically by LINDEMANN[10] and although the first attempt to apply the method, made by ASTON[11] in 1919 with neon, was unsuccessful, it is of considerable historical interest for it led to the discovery of isotopes. Neon has an atomic weight of 20.200 and was the lightest element of which the atomic weight was known with certainty not to be a whole number. In 1913, when J. J. THOMSON was studying the positive radiation produced in neon, he discovered in addition to the predicted mass of 20, a line corresponding to mass 22 which he initially ascribed to an impurity. ASTON then tried to separate these two gases of mass 20 and 22 by fractional adsorption on active carbon cooled by liquid air. The apparatus consisted of a series of empty tubes and tubes filled with active carbon arranged so that the gas proceeded by a series of successive adsorptions and desorptions. After a long and troublesome process corresponding to about 3,000 fractionations, the density of the neon had not altered beyond the precision of the measurements (*ca.* 1%). ASTON concluded that naturally occurring neon consists of a mixture of two gases of mass 20 and 22 respectively, which are completely inseparable by the usual methods; these gases are isotopes.

The only positive results obtained by fractional adsorption are those of GLUECKAUF[1], who in 1949 studied the separation of neon isotopes by adsorption chromatography on active carbon at the temperature of liquid nitrogen. A tube of length 25 cm and diameter 0.7 cm, was filled with active carbon and immersed in a Dewar flask of

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liquid nitrogen; the tube was connected through a capillary with a vacuum pump and to a sampling tube for the subsequent analysis by mass spectrometry (Fig. 2). Several types of test were carried out by varying the experimental conditions and in most cases boundary migration was involved. The head fraction showed an enrichment in ^{20}Ne which could be as high as 15%. By applying the equations of his theory, GLUECKAUF found that the height of the theoretical plates of his column was about 0.2 mm and he deduced the separation factor $\alpha = 1.002$.

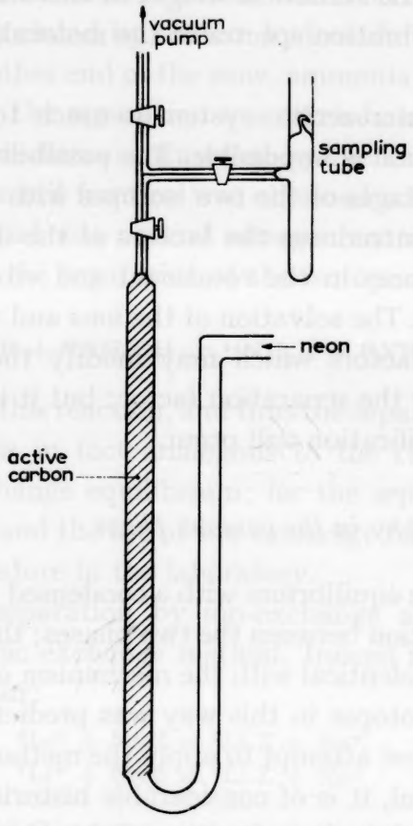

Fig. 2. Chromatography of neon on active carbon.

More recently, GLUECKAUF AND KITT[12] described a method for the complete separation of hydrogen isotopes by gaseous chromatography, in which a dispersion of palladium black in asbestos was used as the adsorbent. The adsorption coefficient decreases as the atomic weight increases (H > D > T) and the only gas which can displace H, D or T from palladium is hydrogen itself. The method used involved boundary migration and it was possible to obtain head fractions in which the heavy isotopes of hydrogen were completely separated. The operation was conducted at ordinary temperatures, but the separation factor decreased when the temperature was raised.

3. ELECTROPHORESIS

The migration of charged particles under the influence of an electric field is a statistical phenomenon associated with diffusion and can be classified generally with the

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diffusion of particles under the influence of an external field. Electrophoresis superimposes a uni-directional movement on the normal random movements of the ions caused by thermal agitation. If a space is considered to be simplified so that displacement is only possible along one axis, the concentration C of the particles varies along this axis and is a function of time t according to the partial derivative equation, a generalisation of FICK's law:

$$\frac{\partial C}{\partial t} = \mathrm{D}\frac{\partial^2 C}{\partial x^2} + V\frac{\partial C}{\partial x} \qquad (1)$$

where D is the diffusion coefficient and V the velocity imparted to the particles along the x-axis by the electric field; $V = E \cdot v$ where E is the electric field and v the mobility of the ions.

This equation describes the general movement of particles in an electric field. The particular integrals possible vary according to the working conditions and are especially dependent on whether the process involves zone electromigration or countercurrent electromigration. The separation of two types of particle is the result of a separation caused by a difference in the diffusion coefficients, and a more efficient separation caused by the difference in mobility. These two quantities v and D vary in the same sense according to a formula described by EINSTEIN in 1905, provided that the basic mechanisms of the particle displacements are the same in both cases:

$$\frac{v}{D} = \frac{\mathrm{e}}{kT}$$

For two isotopes, the elementary application of the kinetic theory of gases, $D\sqrt{M} = C$, shows that the relative difference in mobility is connected with the difference in mass ΔM according to the formula:

$$\frac{\Delta v}{v} = \frac{\Delta D}{D} = \frac{\Delta M}{2M}$$

This relationship is actually only valid in the case of perfect gases. The work which is described below shows that the isotopic effects are much smaller in liquid medium because of the interaction of the ions with the particles of the surrounding medium (*e.g.* solvation).

(*a*) *Electromigration of a zone of two isotopes*

In the case of a zone which can be displaced freely in an undefined medium, the movement of the ions proceeds by a simple, straightforward transference. In fact, if the change of variable $X = x - Vt$ is made in the partial derivative equation (1), the equation becomes an ordinary FICK equation:

$$\frac{\partial C}{\partial t} = D\frac{\partial^2 C}{\partial X^2}$$

This shows that the final partition of the ions is exactly the same as in the case of pure diffusion, but that the whole is displaced in the direction of the electric field by a distance $l = Vt$. However, in practice the widening of the zone during its migration

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is not caused by diffusion alone, for this effect is very small, but is mainly provoked by the inhomogeneity of the diffusion medium and of the electric field.

In the case of a mixture of two isotopes, migration leads to two zones at a distance Δl apart in such a way that $\Delta l/l = \Delta v/v$. It should immediately be noted that the distance l must be measured in relation to the diffusion medium; if the latter is displaced, for example by electro-osmosis, its displacement must be taken into account. However, in the examples described below, the effect of electro-osmosis is small and can be neglected.

Experimentally, the relative difference in mobility is determined in the following way. After the migration, the zone containing the two isotopes is cut as a series of samples, and the concentrations C_1 and C_2 of the two isotopes, and thus the value of the isotopic ratio, are determined in each sample. The distribution curves of each of the two isotopes can then be calculated; their centres of gravity are a distance Δl apart such that $\Delta l/l = \Delta v/v$, l being the total distance travelled by the zone under the influence of the electric field. This method has the advantage that $\Delta v/v$ can be measured precisely and directly without the necessity of a full interpretation of the experimental results. In the Nuclear Chemistry Laboratory of the Collège de France a series of experiments has been carried out in order to establish the relative difference in mobility of two isotopic ions in various salts in the solid or fused state or in aqueous solution. In this way, the mass effect $\mu = \frac{\Delta v/v}{\Delta M/M}$ in the different media could be ascertained and the variation in μ for ions of different mass M could be studied.

(1) *Electromigration in single crystals.* CHEMLA AND SUE[13] observed that isotopic enrichment occurred during the migration of a ^{22}Na–^{24}Na mixture in single crystals of sodium chloride in an electric field at high temperatures. The crystals used were 12 mm long and 3 mm thick, and a thin layer of (^{22}Na + ^{24}Na)Cl was deposited on their surfaces by sublimation. Then each crystal was locked between two carbon electrodes and placed in an electric furnace at 750°, and a potential of 10 volts was applied for 20 hours. Finally the crystals were cut into slices 0.08 mm thick by means of a microtome, the radioactivity of each slice was measured, and the $^{22}Na/^{24}Na$ ratio determined by a study of the radioactive decay. The ratio varied from 1.2 at one end to 1.7 at the other end of the radioactive zone; from these results, the ratio $\Delta v/v$ could be evaluated as 4%.

Analogous results were obtained when ^{22}Na and ^{24}Na migrated in crystals of potassium chloride as well as in a pastille of potassium chloride powder compressed at 5 t/cm^2. In the latter case, the isotopic enrichment was smaller, probably because of intergranular migration.

(2) *Electromigration in aqueous solution.* The first trials of electrophoresis were made by KENDALL[14] who attempted to separate lithium isotopes by electromigration in an agar gel. The attempt was unsuccessful, doubtless because the method used for the isotopic analysis by atomic weight determination was not sufficiently sensitive.

ARNIKAR AND CHEMLA[15] have since repeated the work of KENDALL. Electrophoresis was carried out in a glass tube of diameter 1 cm and length 1 m filled with a

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2.5% gel of Bactogar containing 1% of ammonium nitrate. The initial zone contained 10 mg of lithium nitrate in 1 ml of gel. A potential of 150 volts was applied for 24 hours. At the end of this time, the gel was extruded and cut into 5-mm samples. The lithium zone had travelled 67 cm as determined by flame photometry, and mass spectrometric measurements showed that the $^{7}Li/^{6}Li$ ratio varied from 8.8 at one end of the zone to 15.2 at the other end. The relative difference in mobility ($\Delta v/v$) was thus equal to 0.0036.

The classical technique of paper electrophoresis is the most convenient method for studying electromigration in solution and has been used by BONNIN, CHEMLA AND SUE[16] for the separation of ^{22}Na and ^{24}Na. Since the difference in mobility of ^{22}Na and ^{24}Na is very small, a long distance of migration is necessary and an apparatus was designed in which the ions could cover long distances in a reasonably short time of the order of several hours. A potential of 5,000 volts was applied; to prevent heating effects, the paper was immersed in carbon tetrachloride. As the radioactive zone migrated, the paper strip was simultaneously shifted in the opposite direction, so

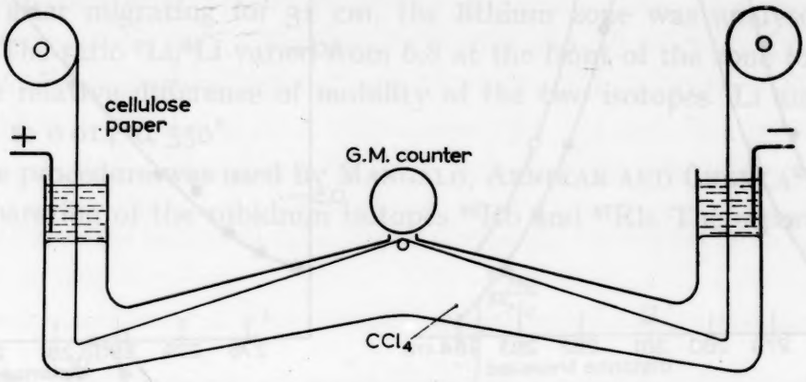

Fig. 3. Electromigration in aqueous solutions over long distances.

that it was possible to travel a distance of several metres in an apparatus of size limited to 40 cm. The position of the radioactive zone was followed during the experiment by means of a Geiger-Müller counter (Fig. 3).

In an experiment carried out at room temperature, the radioactive zone migrated 280 cm in 1 h 45 min. The isotopic ratio $^{22}Na/^{24}Na$, measured by the decrease in radioactivity, varied from 0.28 at one end of the radioactive zone to 1.83 at the other end. From these measurements, it was possible to construct individual distribution curves for ^{22}Na and ^{24}Na, which were about 1 cm apart (Fig. 4). The relative difference in mobility of ^{22}Na and ^{24}Na could thus be calculated as $\Delta v/v = 0.003$. This value is very considerably smaller than that deduced from the law $v\sqrt{M} = C$, even if it is assumed that the Na^{+} ions are hydrated. Similar results are regularly found in work on migration in liquid media.

The effect of hydration of ions can be demonstrated by studying the variation of the isotopic effect with temperature. BONNIN[17] designed an apparatus the principle of which is similar to that of Fig. 3, but in which the carbon tetrachloride was maintained at constant temperature by means of a thermostat. BONNIN found that the

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relative difference in mobility increased very notably when the temperature was raised, the values increasing from 0.003 at 1° to 0.008 at 49°. Thus it can be seen that the structure of the aqueous solution is altered between 0° and 50°, or rather that the hydration of Na^+ ions decreases as the temperature increases.

Paper electromigration has been applied to the separation of lithium isotopes by BONNIN AND CHEMLA[18], who used a strip of Durieux paper, 85 × 4 cm, impregnated with a 10% solution of ammonium nitrate and cooled by immersion in carbon tetrachloride. After electromigration for 14 h 30 min at a potential of 300 V, the zone had

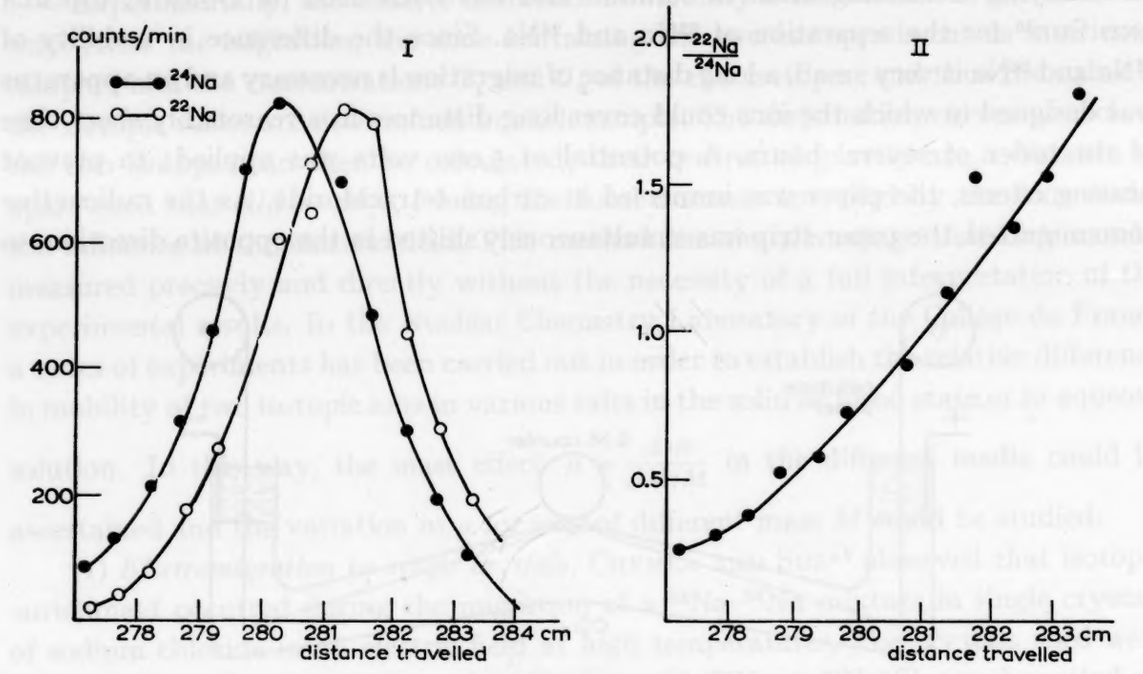

Fig. 4. Electromigration of ^{22}Na–^{24}Na mixtures in aqueous solution. I: Separation of ^{22}Na and ^{24}Na. II: The ratio ^{22}Na/^{24}Na along the radioactive zone.

travelled 56 cm. The isotopic ratio, determined by mass spectrometry, varied from ^{7}Li/^{6}Li = 7.6 at the head of the zone to ^{7}Li/^{6}Li = 15.0 at the tail. The distribution curve of the total lithium was determined by isotopic dilution with pure ^{6}Li and the relative difference in mobility of ^{6}Li and ^{7}Li was thus found equal to $\Delta v/v = 0.0038$.

(3) *Electromigration in fused salts.* The technique of zone electromigration can be extended to fused salts. A porous support is necessary to prevent convection currents; among the possible materials, asbestos paper is most advantageous, for the method then becomes quite similar to classical electrophoresis on cellulose paper. Although it appears more complicated on account of the high temperature required, it is possible to pass a much stronger electric current without trouble and approximately 10 times the amount of material can be dealt with.

Electrophoresis is carried out on strips of asbestos paper, 62 cm long, 12 mm wide and 0.3 mm thick, impregnated with fused alkali metal nitrates; the initial zone consists of about 20 mg of the salt under examination. The operation takes place in a horizontal furnace made from a Pyrex tube heated electrically to 350° (Fig. 5). An

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applied potential of 600 V and a current of 200 mA are used. Under these conditions, the ions travel a distance of some 30 cm in 2–3 hours.

This method was first used by CHEMLA AND BONNIN[19] for the separation of ^{22}Na and ^{24}Na. With a potential gradient of 14 V/cm, the $^{22}Na + {}^{24}Na$ zone migrated 35 cm

Fig. 5. Electromigration on asbestos paper impregnated with fused salts.

in 4 hours. The isotopic ratio varied from 0.6 at one end of the zone to 1.8 at the other end and the relative difference in mobility was found to equal 0.01 (Fig. 6).

Later CHEMLA[20] showed that the separation of lithium isotopes could be studied very easily by this method, since appreciable amounts of material can be used for electrophoresis in fused salts. The initial zone consisted of 30 mg of pure lithium nitrate and, after migrating for 31 cm, the lithium zone was analysed by flame photometry. The ratio $^{7}Li/^{6}Li$ varied from 6.8 at the front of the zone to 14.7 at the rear, and the relative difference of mobility of the two isotopes ^{7}Li and ^{6}Li ($\Delta v/v$) corresponded to 0.014 at 350°.

The same procedure was used by MANGALO, ARNIKAR AND CHEMLA[21] in order to study the separation of the rubidium isotopes ^{85}Rb and ^{87}Rb. The separation of the

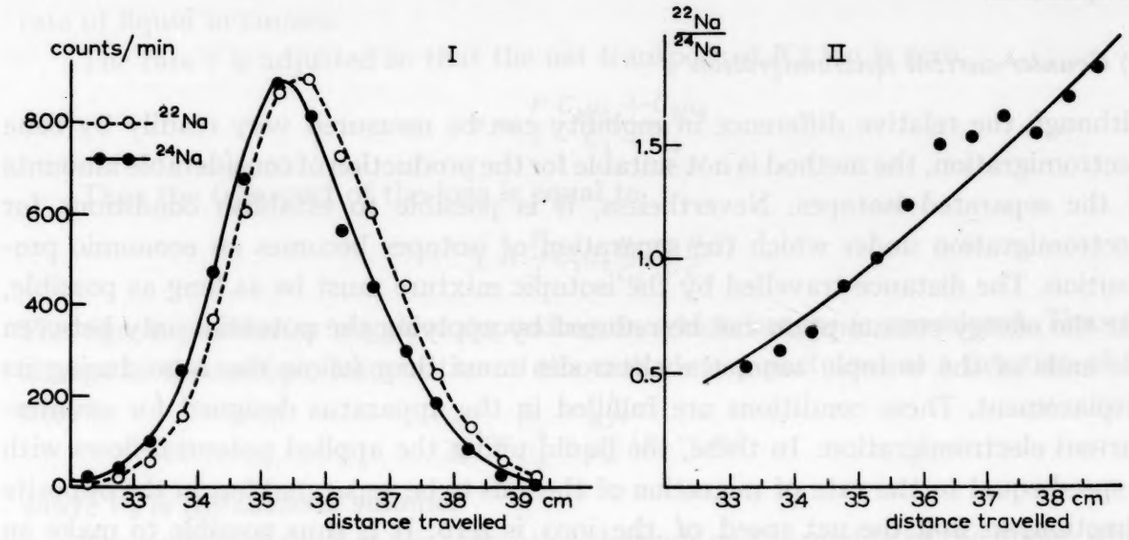

Fig. 6. Electromigration of ^{22}Na–^{24}Na mixture in $NaNO_3$. I: Separation of ^{22}Na and ^{24}Na. II: The ratio $^{22}Na/^{24}Na$ along the radioactive zone.

final zone was determined by means of the radioactive indicator ^{86}Rb. The ratio $^{85}Rb/^{87}Rb$ was measured by mass spectrometry and had values of 3.00 at the front of the zone and 2.40 at the rear; the difference in mobility of these two isotopes was found equal to 0.00117.

Finally, ARNIKAR AND CHEMLA[15] have applied the technique to the separation

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of the radioactive isotopes ^{131}Cs and ^{137}Cs. The isotopic ratio was measured by the analysis of the β-rays emitted by means of an absorbent screen. After a migration of 41 cm, the ratios $^{131}Cs/^{137}Cs$ at the front and rear of the radioactive zone were 0.59 and 0.35 respectively. The difference in mobility ($\Delta v/v$) was 0.0027.

The results obtained by electromigration in fused sodium nitrate are summarized in Table 1.

TABLE 1

Isotopes	$\Delta v/v$	$\mu = \Delta v/v : \Delta M/M$
^{6}Li–^{7}Li	0.014	0.089
^{22}Na–^{24}Na	0.010	0.10
^{85}Rb–^{87}Rb	0.0016	0.069
^{131}Cs–^{137}Cs	0.0027	0.06

These results show that $\Delta v/v$ depends not only on the relative difference in mass but also on the absolute mass of the isotopes under consideration. This variation can be qualitatively explained by a kinetic theory provided that the following points are taken into account. In an electric field, the lightest ions undergo the greatest acceleration, but, on the other hand, the heaviest ions are the least retarded by collision with solvent particles and thus the difference in the rates of migration of two isotopes is narrowed. However, the interaction of particles in a condensed medium is a subject of which little is known and the basic mechanism of the migration is certainly very complicated.

(*b*) *Counter-current electromigration*

Although the relative difference in mobility can be measured very readily by zone electromigration, the method is not suitable for the production of considerable amounts of the separated isotopes. Nevertheless, it is possible to establish conditions for electromigration under which the separation of isotopes becomes an economic proposition. The distance travelled by the isotopic mixture must be as long as possible, but the energy consumption can be reduced by applying the potential only between the ends of the isotopic zone; the electrodes must then follow the zone during its displacement. These conditions are fulfilled in the apparatus designed for counter-current electromigration. In these, the liquid under the applied potential flows with a speed equal to the rate of migration of the ions to be separated but in the opposite direction, so that the net speed of the ions is zero. It is thus possible to make an isotopic mixture traverse a very long effective distance in an apparatus of modest dimensions. Some extremely interesting work based on this principle has been carried out in the U.S.A. and in Germany; the former used salts in aqueous solution whereas the latter worked with fused salts.

(1) *Counter-current electromigration in aqueous solutions.* This technique for the separation of isotopes was developed in 1941 but remained a defence secret until 1946 when the first publication of BREWER, MADORSKY AND WESTHAVER[22] on the separa-

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tion of potassium isotopes appeared. In a subsequent paper, BREWER, MADORSKY *et al.*[23] described the technique of their method in detail. The counter-current principle is as follows. A U-tube, filled with sand in the horizontal part, contains a solution of potassium sulphate. When a potential difference is applied between the ends of the tube, the K^+ ions move towards the cathode and the SO_4^{--} ions towards the anode. Total reflux can be ensured by adding sulphuric acid to the cathode compartment in such an amount and concentration that the pH remains neutral and the concentration of the cathode solution is unchanged. Under these conditions, the flow of electrolyte in the porous packing exactly compensates the rate of migration of the K^+ ions. As $^{39}K^+$ ions have a slightly greater mobility than $^{41}K^+$ ions, the former can make headway against the current whereas the latter are carried towards the anode compartment. Thus the cathodic compartment is enriched in ^{39}K. The anode compartment is constantly supplied with potassium hydroxide in order to compensate for the formation of sulphuric acid and its isotopic composition does not alter.

These methods can be used to determine the difference in mobility of two isotopes by means of the observed isotopic enrichments.

If C_1 and C_2 are the concentrations of the isotopes ^{39}K and ^{41}K whose mobilities are μ_1 and μ_2, the rate of transport of the ions across the actual cross-section area A of the packing is:

$$AC_1\left(\mu_1\frac{P}{l}-v\right)\text{ ions }^{39}\text{K},\quad\text{and}\quad AC_2\left(\mu_2\frac{P}{l}-v\right)\text{ ions }^{41}\text{K}$$

where P is the applied potential difference, l the length of the packing, and v the flow rate of liquid in cm/sec.

The rate v is adjusted so that the net transport of K^+ ion is zero,

$$v=\frac{P}{l}\frac{C_1\mu_1+C_2\mu_2}{C_1+C_2}$$

Thus the transport of the ions is equal to

$$\pm A\frac{P}{l}C_1C_2\frac{\mu_1-\mu_2}{C_1+C_2}$$

The sign is positive or negative depending on which isotope is considered. Thus the change of the isotopic composition of the cathode compartment as a function of the time is

$$\frac{\mathrm{d}}{\mathrm{d}t}\log\frac{C_1}{C_2}=\frac{AP}{V_cl}(\mu_1-\mu_2)$$

where V_c is the cathode volume.

The success of the isotopic separation depends on the quality of the filling, which constitutes the diaphragm, on the nature of the electrolyte, and especially on the precision with which it is ensured that the rate of migration of the ions and the flow rate of the liquid are in exact equilibrium.

The apparatus used for this work is shown schematically in Fig. 7.

BREWER AND MADORSKY made a systematic study of the factors influencing the separation and found that the efficiency of the separation is virtually unaffected by the size of the separation tube, the nature of the filling, the anion in combination with

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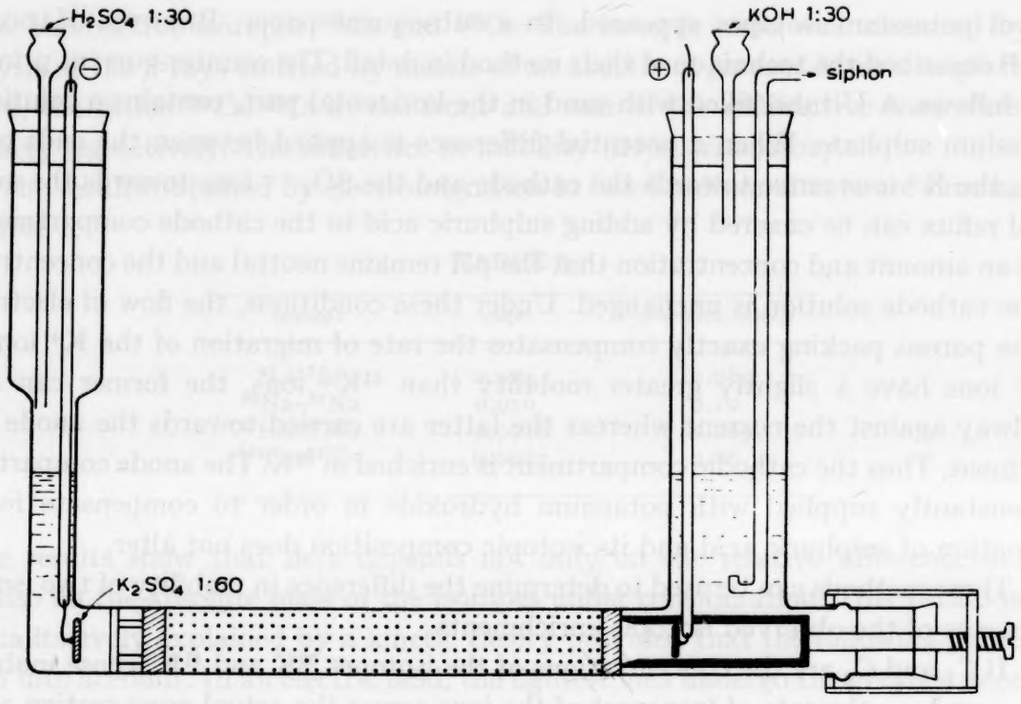

Fig. 7. Apparatus of BREWER AND MADORSKY for the separation of ^{41}K by counter-current electromigration.

potassium, the electrolytic current, and the temperature. The main source of trouble arises from lack of uniformity in the porous medium which may cause channelling produced by the liberation of air bubbles within the packing. Among the various materials which may be used for the packing, fine sand (100 mesh) is said to be considerably better than sintered glass, glass wool, asbestos or capillary tubes.

The apparatus is of the type shown in Fig. 7, and the separation tube of length 10 cm and diameter 1.4 cm is filled with fine sand. The separation of potassium isotopes is carried out in the following way. The tube is filled with an aqueous 1:60 potassium chloride solution while the cathode compartment is supplied with a 1:30 hydrochloric acid solution so that the pH remains neutral, and the anode compartment is supplied with a 1:30 potassium hydroxide solution. The apparatus is placed in a thermostat. A potential of 93 V is applied with a current of about 0.5 A. The cathode has a volume of 11 cm^3. At intervals the isotopic composition of the cathode compartment is measured by mass spectrometry (Table 2). BREWER AND MADORSKY deduced that the relative difference of mobility of ^{39}K and ^{41}K was in the neighbourhood of 0.0022.

TABLE 2

Hours	$^{39}K/^{41}K$
0	14.20 (natural K)
41	15.30
131	16.4
209	19.2
377	22.2
449	24.0

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The apparatus described in Fig. 7 is only useful for the production of fractions enriched in the lighter isotope. An enrichment in ^{41}K can be obtained by modifying the procedure slightly; the anode compartment is filled with lithium chloride while the separation tube is filled with potassium chloride. The boundary between the two solutions is clear and remains stable throughout the experiment; it becomes very sharp when the potential is applied because of the lower mobility of the lithium ions. The anode compartment is constantly supplied with lithium chloride in order to eliminate the products of electrolysis. The $^{39}K/^{41}K$ ratio can be reduced to 9.1 by this method.

The above apparatus has also been used for the separation of isotopes by migration of anions. MADORSKY AND STRAUS[24] separated chlorine isotopes in this way;

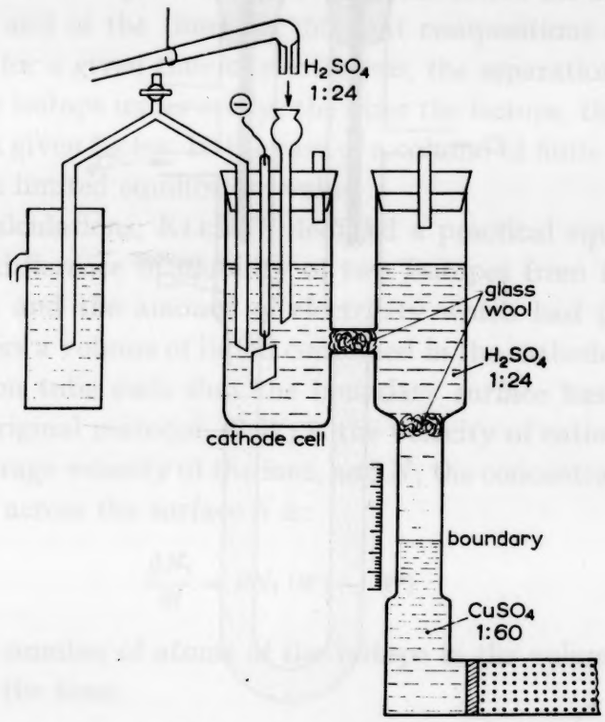

Fig. 8. Apparatus for concentration of ^{63}Cu. The boundary between $CuSO_4$ and H_2SO_4 prevents the deposit of copper on the cathode.

variations in the $^{35}Cl/^{37}Cl$ ratio of up to 30% were obtained. Chemical reactions which may interfere with the operation can be prevented by the use of boundaries which separate the tube from the electrode compartments. Thus for the separation of copper isotopes, MADORSKY AND STRAUS[25] maintained in the vicinity of the cathode compartment a sharp boundary between the copper sulphate solution and a solution of sulphuric acid (Fig. 8). The isotopic enrichments scarcely attained 1–2%; for the difference in mobility of ^{63}Cu and ^{65}Cu seems to be extremely small. In a similar way, boundaries have been used in the separation of uranium isotopes with uranyl nitrate as electrolyte; an enrichment in ^{235}U of 2.8% was obtained. A patent[26] has been obtained which covers all these results.

Finally it is worth noting a paper by RAMIREZ[27] who obtained slight isotopic enrichment with rubidium by counter-current electromigration in an apparatus

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similar to that of BREWER AND MADORSKY; the packed tube was replaced by a tight stack of semipermeable membranes. RAMIREZ states that he observed a slight temperature effect which he attributed to a variation in the hydration of the ions.

(2) *Counter-current electromigration in fused salts.* During 1944 in Germany, KLEMM[28] suggested that the separation of isotopes by electromigration would be more efficient in "dry" media; for in solution the relative difference in mass of the isotopic ions is decreased by solvation phenomena. Initially, KLEMM used solid salts. For example, when a solid bar of α-AgI underwent prolonged electrolysis at 230°, the

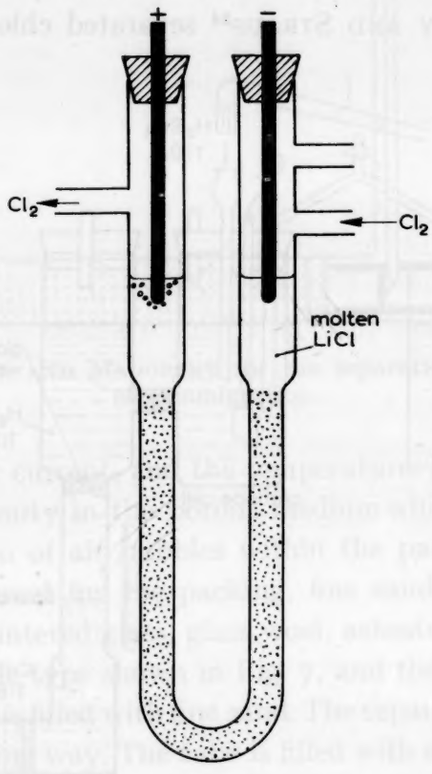

Fig. 9. Apparatus used by KLEMM for the separation of lithium isotopes. Chlorine is admitted at the cathode to prevent the deposit of metallic lithium.

boundary between the carbon electrode and the silver iodide became progressively enriched in ^{109}Ag, the concentration of which increased from 48% to 51%[29].

Later, in 1947, KLEMM[30] turned his attention to fused salts and developed a method of counter-current electromigration by which he was able to obtain considerable isotopic enrichment with lithium. A very simple apparatus used for the separation of lithium isotopes[31] is shown schematically in Fig. 9. The tube is made of Supremax glass and is filled with lithium chloride maintained at 650°. The U-part of the apparatus is the separation tube and is 5.6 mm in diameter and filled with granular quartz of grain size 0.13 mm. Carbon electrodes and a 0.5 A current are used. During electrolysis, chlorine is liberated at the anode while the metallic lithium deposited on the cathode is immediately converted to lithium chloride by a stream of chlorine. The counter-current is thus ensured and this is one advantage of using fused salts. Further-

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more, since the tube always contains pure lithium chloride, the conditions for total "reflux" are always fulfilled; the liquid levels on the two sides of the separation tube are kept equal by regulating the nitrogen pressure on the cathode compartment. With such an apparatus, one gram of lithium chloride whose ^6Li content had altered from 7.3 to 16.1% was obtained after four days.

The theory of the mechanism of isotopic enrichment by this process is clearly analogous to that of the methods of BREWER AND MADORSKY. KLEMM[32] has made a theoretical treatment of the general case of the separation of a binary mixture in a column of infinite length. After laborious calculation, he was able to construct a graph which gave the value of the separation factor as a function of the difference in mobility of the two isotopes and of the time, for different compositions of the mixture. He then observed that, for a given time of electrolysis, the separation factor depends on the proportion of the isotope under study; the rarer the isotope, the more difficult it is to concentrate it to a given factor. In the case of a column of finite length, the isotopic enrichment attains a limited equilibrium value.

By simplified calculations, KLEMM[33] deduced a practical equation by which he could calculate the difference in mobility of two isotopes from the analyses of the isotopic composition and the amount of electricity which had passed through the tube. KLEMM considers a volume of liquid contained in the cathode compartment and part of the separation tube such that the boundary surface has the same isotopic composition as the original material. If W_i is the velocity of cation i relative to that of Cl ions, W^0 the average velocity of the ions, and N_i the concentration of the isotope, the rate of transport across the surface S is:

$$\frac{dN_1}{dt} = SN_1 (W_1 - W^0)$$

where N_i is the total number of atoms of the isotope in the volume considered.

As a function of the time:

$$N_1 = N^0{}_1 - SN^0{}_1 (W_1 - W^0)\, t$$

On the other hand, the electric charge transported by the cations across the same surface S is: $Q = S \cdot F \cdot N^0 \cdot W^0 t$ (F = Faraday).

These two equations give:

$$\frac{W_1 - W^0}{W^0} = \frac{N_1{}^0 - N_1}{N_1{}^0} \cdot \frac{FN^0}{Q}$$

And if these equations are given for two isotopes i and j, it can be deduced that:

$$\frac{W_1 - W_j}{W^0} = -\left(\frac{N_1}{N_1{}^0} - \frac{N_j}{N_j{}^0}\right)\frac{FN^0}{Q}$$

From this equation the relative difference in mobility $\frac{W_1 - W_j}{W^0}$ of the two isotopes can be deduced. Quite recently, KLEMM AND MONSE[34] have extended this method for the general measurement of the mobilities of various ions in a mixture of fused salts. These mobilities can be measured directly by observation of the moving boundaries.

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However, they can also be deduced from chemical and isotopic analysis of the anode and cathode compartments; a calculation similar to that above can be applied to any mixture of isotopes or different ions.

The method of KLEMM has been used in a large number of experiments for the determination of the differences in mobility of isotopes in fused salts. The apparatus

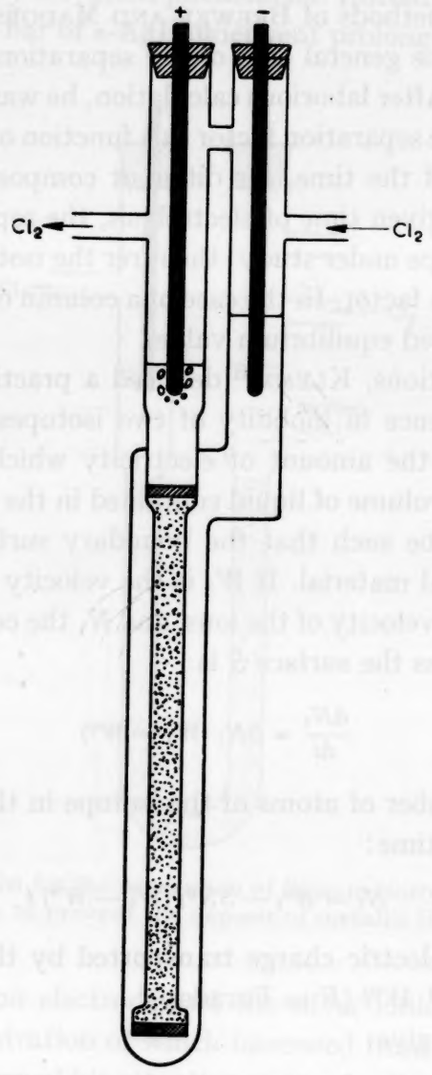

Fig. 10. Counter-current electromigration in fused metallic halides.

used is shown in Fig. 10. The separation column is straight, made from Supremax glass, and filled with glass powder held in position by sintered glass discs; it separates the anode compartment from the cathode compartment. The apparatus is placed in a vertical furnace maintained at an appropriate temperature. This technique is especially suitable for the separation of halogen isotopes. Thus, for the separation of chlorine isotopes, KLEMM AND LUNDEN[35] used molten lead chloride as electrolyte. Since the anode was made of lead, it dissolved constantly during the electrolysis to reform lead chloride; total reflux was always ensured. After electrolysis for seven

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days, the concentration of ^{35}Cl in the anode compartment had doubled; the difference in mobility of ^{35}Cl and ^{37}Cl was found equal to 0.29%. In this technique, lead chloride can be replaced by zinc chloride in which case the anode is made of zinc[36]. Similarly, lead bromide can be used as electrolyte for the separation of bromine isotopes[37].

In the separation of isotopes of electropositive elements, reflux is ensured by continual recombination of the metal which is deposited on the cathode. The electrolyte is most often a metallic chloride so that the metal salt can be regenerated by bubbling chlorine through the cathode compartment. This technique has been used for the separation of lithium isotopes[31], for the recombination of the alkali metals proceeds very readily. For other metals, it is advantageous to form a "boundary" with a second salt between the separation tube and the cathode compartment. For example, KLEMM, HINTENBERGER AND LUNDEN[38] separated zinc isotopes by electrolysis of fused zinc chloride, and avoided the deposit of zinc by placing a reservoir of lead chloride between the separation tube and the cathode. Thus only metallic lead was deposited on the cathode; the boundary between the lead chloride and zinc chloride remained sharp throughout the operation. This technique is improved further by placing lithium chloride in the cathode compartment; the lithium deposited on the cathode is constantly recycled as lithium chloride by chlorine bubbling through in the vicinity of the cathode. As the cation Li^+ is very mobile, the boundary between the lithium chloride and the second chloride remains sharp even during prolonged electrolysis. In this way, FLOBERG, KLEMM AND LANG[39] obtained a partial separation of silver isotopes. A constant current of 300 mA was passed through the chain: carbon anode | fused AgCl | fused LiCl | carbon cathode; after 50 hours, the ratios $^{107}Ag/^{109}Ag$ at the ends of the separation tube differed by about 10%

The method is thus applicable to other elements and partial separation of copper isotopes[40] or lead isotopes[41] is possible by prolonged electrolysis of the corresponding chloride with a cathode of constantly regenerated lithium chloride.

LUNDEN[42] has studied the effect of the nature of the anion. He found that the separation of lithium isotopes is more efficient when lithium bromide is used. The relative difference in mobility of ^6Li and ^7Li is about twice as great in lithium bromide as in lithium chloride.

Since the metallic halides generally have high melting points, which makes it difficult to obtain practicable materials for the construction of the separation tubes, LUNDEN tried to apply the technique of counter-current electromigration to fused nitrates. However, it is then difficult to ensure regeneration of the metallic oxides which are deposited on the cathode. Only partial recombination is obtained by bubbling through it a mixture of oxygen and nitrogen dioxide. In these tests slight separations of the isotopes of potassium[43] and lithium[44] were observed.

The preceding experiments have allowed the relative difference in mobility $\Delta v/v$ of isotopic ions of various elements to be measured. In order to make the numerical results comparable, the mass effect should be introduced:

$$\mu = \frac{\Delta v}{v} \Big/ \frac{\Delta M}{M}$$

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which is independent of the relative difference in mass $\Delta M/M$. The results of the measurements are collected in Tables 3 and 4. These tables show that the mass effect varies with the atomic weight of the element studied as well as with the atomic weight of the element combined with it.

TABLE 3

EFFECT OF MASS IN ELECTROMIGRATION OF METALS

Isotopes	*Fused medium*	*Effect of mass*
Li	LiCl	0.14
Li	LiBr	0.26
Li	$LiNO_3$	0.05
Zn	$ZnCl_2$	0.078
Zn	$ZnBr_2$	0.11
K	KNO_3	0.037
Cu	CuCl	0,080
Ag	AgCl	0.064
Cd	$CdCl_2$	0.067
Tl	TlCl	0.040
Pb	$PbCl_2$	0.024

TABLE 4

EFFECT OF MASS IN ELECTROMIGRATION OF HALOGENS

Isotopes	*Fused medium*	*Effect of mass*
Cl	$ZnCl_2$	0.043
Cl	TlCl	0.086
Cl	$PbCl_2$	0.052
Br	$PbBr_2$	0.042

A simplified kinetic theory indicates that the mobility is directly proportional to $1/\sqrt{M}$ and that the mass effect is constant for all elements. However, the experimental results show that a more complete theory must take into account the relative mass of the isotopic ions as well as the solvent particles. KLEMM has attempted to explain the mechanism of migration by attributing a quasi-crystalline structure to the liquid medium so that the displacement of an ion proceeds from one position to the next by a series of "jumps". However, for the theoretical values to confirm the practical results, KLEMM[45] admits that the jumps could be of two types: "spontaneous jumps", the frequency of which depends on the relative masses of the migrating particles and on the solvent molecules, and "induced jumps" independent of mass and provoked by the agitation of neighbouring particles.

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